DEHYDRATION INCREASES L-TYPE CALCIUM CHANNEL DENSITY IN THE SOMATA OF MAGNOCELLULAR NEUROSECRETORY CELLS IN RATS

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By

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ABSTRACT

The magnocellular neurosecretory cells (MNCs) of the hypothalamus are responsible for the synthesis and secretion of vasopressin (VP), which is important for fluid homeostasis, and oxytocin (OT), which is responsible for uterine contraction during parturition and milk let-down during lactation. VP-ergic MNCs undergo a number of structural and functional changes during dehydration, including the adoption of a bursting pattern of firing, the retraction of glial processes from MNC somata and terminals, the translocation of κ-opioid receptors from internal stores to the plasma membrane, and the somatodendritic release of VP and OT. Since voltage-gated Ca^{2+} channels have been found on intracellular granules, and since an increase in Ca^{2+} current could regulate firing patterns and neuropeptide release, the surface expression of Ca^{2+} channel subtypes in MNCs was tested to determine if it would be altered by 16-24 hours of water deprivation. Using radioligand binding of antagonists of N-type and L-type Ca^{2+} channels, channel density was measured in the supraoptic nucleus (SON), which is largely composed of MNC somata, and in the neurohypophysis (NH), which is largely composed of MNC terminals. Dehydration caused an increase in the density of L-type channels in the SON, while causing no significant change in the N-type density. No change in density of either channel type was observed in the NH. Electrophysiological measurements in isolated MNC somata showed no change in total Ca^{2+} current, but a significant increase in the nifedipine-sensitive current following dehydration. Reverse transcription-polymerase chain reaction (RT-PCR) demonstrated no increase in messenger RNA levels for L-type channels, suggesting that the increase in channel density is not a consequence of *de novo* synthesis. These results suggest that L-type Ca^{2+} channels may be translocated from internal stores to the plasma membrane of MNCs in response

to dehydration. Such a process may be important in maximizing secretion of VP when the physiological need is high.

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Dedication

To Steven, Emily and Romie, for the sacrifices you made as I pursued my dreams.

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LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AP	action potential
ATP	adenosine triphosphate
BCCa	bag cell Ca^{2+} channel
bp	base pairs
BSA	bovine serum albumin
CA1	hippocampal region CA1
CCD	charge-coupled device (digital)
CCK	cholecystokinin
cDNA	complementary DNA
CNS	central nervous system
$Cy_{2/3}$	Cyanine $_{2/3}$
DAP	depolarizing afterpotential
DHP	dihydropyridine
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ELH	egg laying hormone
ER	endoplasmic reticulum
GABA	gamma aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GRC	growth-factor-regulated channel

HB	homogenization buffer
HEK-293	human embryonic kidney cell line
HNS	hypothalamic neurohypophysial system
I_{Ca}	total Ca^{2+} current
IGF-I	insulin-like growth factor I
IMR32	human neuroblastoma cell line
IP_3	inositol trisphosphate
kDA	kilo dalton
KOR1	κ-opioid receptor 1
LDCV	large dense core vesicles
LSV	liquid scintillation vial
MNC	magnocellular neurosecretory cell
MnPO	median preoptic nucleus
mRNA	messenger ribonucleic acid
NH	neurohypophysis
OT	oxytocin
OVLT	organum vasculosum lamina terminalis
PC12	pheochromocytoma cells
PGC	PIPES with glucose and Ca^{2+}
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
PKC	protein kinase C
pS	pico Seimens
PVN	paraventricular nucleus

RER	rough endoplasmic reticulum
RINm5F	rat insulinoma cell line
RT-PCR	reverse transcription-polymerase chain reaction
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SFO	subfornical organ
SIC	stretch-inactivated cation channel
SNARE	soluble NSF attachment protein receptors
SON	supraoptic nucleus
TEA	tetraethylammonium
TRH	thyrotropin releasing hormone
TRP	transient receptor potential
TTX	tetrodotoxin
UV	ultraviolet
V_1R	vasopressin 1 receptor
VDCC	voltage-dependent calcium channels
VP	vasopressin
ω-Ctx-GVIA	ω-conotoxin-GVIA
ω-Ctx-MVIIC	ω-conotoxin-MVIIC

CHAPTER 1
INTRODUCTION

Magnocellular neurosecretory cells

Anatomy and function

The magnocellular neurosecretory cells (MNCs) are neuroendocrine cells responsible for the synthesis and release of vasopressin (VP) and oxytocin (OT) (Bargmann & Scharrer, 1951). As illustrated in Figure 1.1, the somata are located principally in two hypothalamic nuclei, the supraoptic (SON) and paraventricular (PVN), and the terminals are found in the neurohypophysis (NH). There are other MNCs distributed within the hypothalamus as well, but the SON and PVN are responsible for 50% of the MNC cell somata (Hatton & Li, 1998a). Within the NH, other innervating axons (e.g. GABAergic, dopaminergic, noradrenergic, enkephalinergic) are present in detectable amounts, but the neurosecretory axons, axonal swellings, and terminals constitute the overwhelming proportion of neural elements. The somata and terminals can be readily isolated by enzymatic treatment to enable electrophysiological and immunocytochemical study. Figure 1.1 is a cartoon showing the relative positions of the SON and NH and the micrographs are of an acutely isolated soma (upper) and terminals (lower). The physical separation of somatic stimulation from axonal secretion makes the MNCs an excellent model for the study of the events that occur following stimulation-coupled secretion within the same cell.

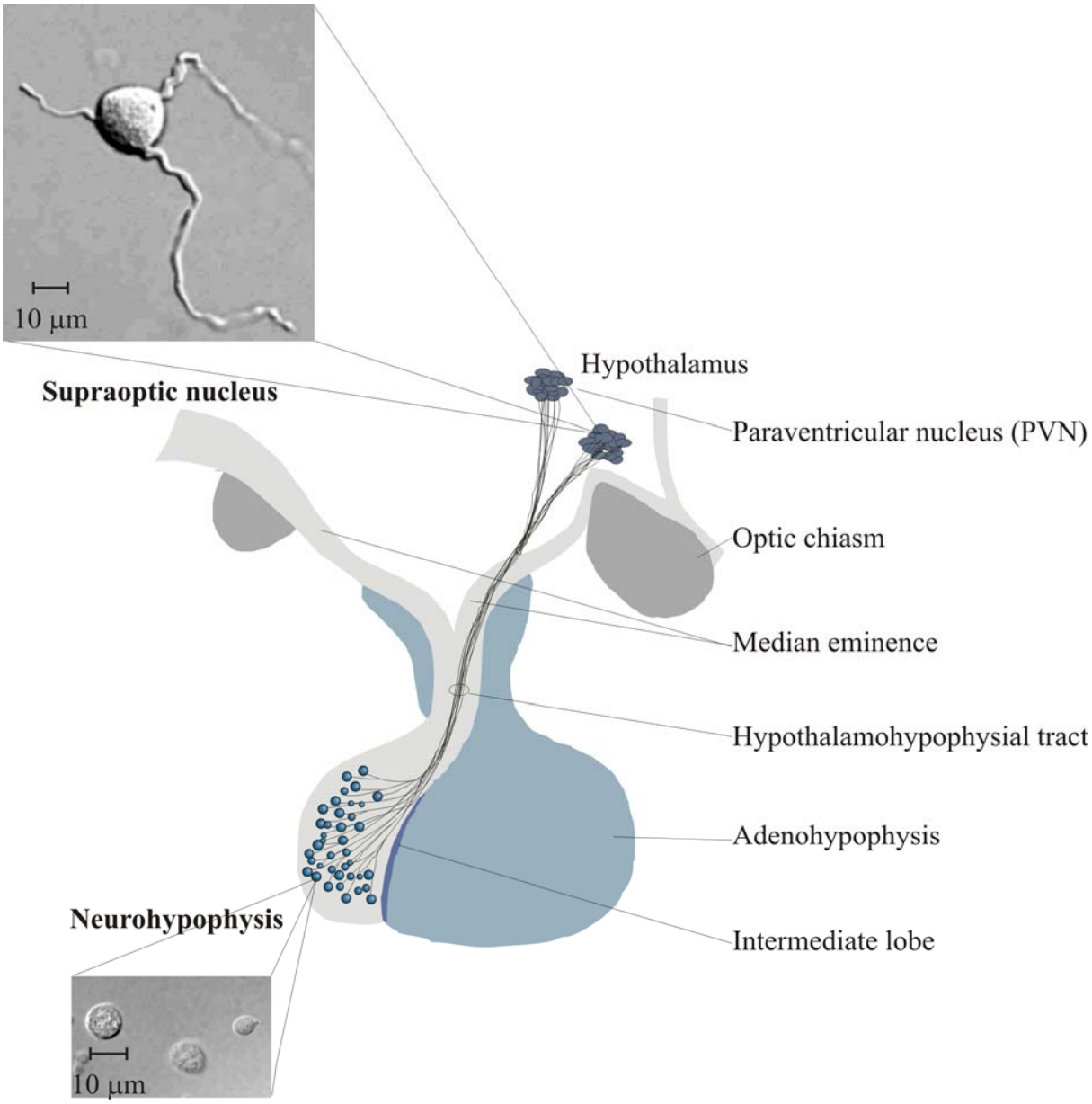

Figure 1.1. Anatomy of the MNCs. The cell bodies are located in two hypothalamic nuclei, the supraoptic and paraventricular, and the terminals are in the neurohypophysis. The inserts are micrographs of an isolated soma (upper) and terminals (lower).

One to three dendrites extend from the MNC somata; toward the subependymal region of the third ventricle for MNCs of the PVN, and toward the ventral glial lamina for cells from the SON (Armstrong, 1995). The dendrites also contain neurosecretory granules that are released into the extracellular space, and release by exocytosis is possible from anywhere on the plasma

membrane (Pow & Morris, 1989). Dendritic release may be important in self-modulating the MNC activity (Ludwig & Pittman, 2003) and this will be discussed later. Each MNC projects a single axon arising from the soma or primary dendrite and the axons from all hypothalamic nuclei collect in the tract of Greving, which eventually leads to the internal layer of the median eminence and infundibular stalk into the NH. Within the NH, there is considerable dilatation and arborisation of the terminals and each axon devolves into 2000-10,000 varicosities containing neurosecretory granules. The largest of these varicosities are called Herring bodies, and these play a major role in granule storage. The axonal terminations or buds near the fenestrated capillaries are the sites of hormone release into the circulation. Pituicytes, modified astroglial cells, engulf the terminal buds, and are responsible for the ultrastructural plasticity seen in OTergic terminals (Theodosis, 2002) and VPergic terminals (Tweedle & Hatton, 1980).

Although the VPergic and OTergic subpopulations can be clearly differentiated morphologically in other mammalian species, they cannot be in rat (Armstrong, 1995). However, they can be differentiated immunocytochemically using antibodies to the hormones (Poulain & Wakerley, 1982) and electrophysiologically (Armstrong & Stern, 1998). When stimulated, OT neurons exhibit relatively short, high frequency patterns of activity and the OT cells are organized within local pattern-generating networks that facilitate synchronous bolus-like release of OT (Leng *et al.*, 1999). When the VPergic neurons are stimulated, they produce a phasic firing pattern, with lower frequency firing for longer periods separated by long silent intervals. Maximum VP release is facilitated through the intrinsic burst-generating properties of these cells (Wakerley *et al.*, 1978). There is some overlap in function as most OT cells also express some VP mRNA and most VP cells contain some OT mRNA, while others express equivalent levels of both OT and VP mRNA (Gainer, 1998).

The primary role of the MNCs is to synthesize and release VP and OT under the appropriate stimulation; VP to maintain fluid homeostasis and OT to stimulate uterine smooth muscle contraction at parturition and contraction of myoepithelial cells within the mammary gland causing milk to be ejected during lactation. Both sexes secrete OT and pulses of OT can be detected during ejaculation. Current evidence suggests that OT is involved in facilitating sperm transport within the male reproductive system and perhaps also in the female, due to its presence in seminal fluid (Studdard *et al.*, 2002). It may also have effects on some aspects of male sexual and social behavior (Landgraf & Neumann, 2004) including trust (Kosfeld *et al.*, 2005). In rats, dehydration also stimulates the release of OT, which is involved in natriuresis and acts on the kidney to increase sodium excretion while also reducing salt appetite (Verbalis *et al.*, 1991). Dehydration and hemorrhage will both induce the release of VP, although through different mechanisms. Hypothalamic osmoreceptors sense extracellular osmolality with high sensitivity and stimulate VP release when osmolality rises by as little as 1%, as occurs with dehydration. The mechanistic details of this response will be discussed later. Hypovolemia, which occurs during hemorrhage, results in a decrease in atrial pressure. Specialized stretch receptors within the atrial walls and large veins (cardiopulmonary baroreceptors) entering the atria decrease their firing rate when there is a fall in atrial pressure. Afferent nerve fibres from these receptors synapse within the nucleus tractus solitarius of the medulla, which sends fibres to the hypothalamus. Atrial receptor firing normally inhibits the release of VP; however, with hypovolemia or decreased central venous pressure, the decreased firing of atrial stretch receptors leads to an increase in VP release. It takes a larger disruption, in the order of a 15-20% decrease in blood volume, for a response (Sladek, 2000). A third stimulus for VP release is central nervous system input mediated by nausea (Kim *et al.*, 1997), pain (Bodnar *et al.*, 1986), hypoxia

(Kelestimur *et al.*, 1997), or pharyngeal stimuli such as vomiting (Koch *et al.*, 1990). Regardless of the stimulant, the hormones that are synthesized in the cell body are packaged in vesicles and transported to release sites. Those destined to have a systemic effect are transported along axons to the terminals for release into the blood, while those required centrally are released directly from the somata and dendrites. The present research focuses on the changes in the MNCs following stimulation by dehydration.

Characteristics of osmosensitivity

Osmoregulation and fluid homeostasis in mammals is vital and deviations from an ideal osmotic pressure can have dire physiological consequences. In order to maintain osmotic pressure at this ideal set point, the physical perturbation is converted into an electrical signal that initiates exocytosis of the hormone and ultimately the downstream effect of restoring fluid balance and plasma osmolality. So important is this that there are multiple pathways for sensing changes and for initiating a response. Within the CNS, the capillaries perfusing the subfornical organ (SFO), the median preoptic nucleus (MnPO), and the organum vasculosum lamina terminalis (OVLT), are highly porous and allow substances in the blood to come in contact with neurons. These neurons are intrinsically sensitive to fluid osmolality (Bourque & Oliet, 1997) and they have afferent inputs to the SON and PVN (Hatton & Li, 1998b). Working in concert with these inputs is the osmosensitivity of the MNC itself (Oliet & Bourque, 1992). In isolated MNC somata, hyperosmolar media causes the cell to become depolarized while hypoosmolar media has the effect of hyperpolarizing the cell. Stretch-inactivated cation (SIC) channels present on the MNC plasma membrane play a role in this response (Oliet & Bourque, 1994). Briefly, in hyperosmolar conditions, the shrinkage of the cell reduces the tension on the channels within the plasma membrane, causing them to open more frequently, and bringing the membrane potential closer to threshold. Conversely, the stretch experienced under hypoosmolar conditions results in

these channels being inactivated and the membrane potential moves away from threshold. In this way, the cell becomes more or less responsive to the afferent neuronal inputs. In the MNC somata, the SIC channels transduce osmotically-evoked changes in cell volume into functionally relevant changes in membrane potential (Bourque & Oliet, 1997). This modulation may be critical for the change in the firing pattern of the VPergic neurons from slow and irregular to phasic bursting that is integral to the maximization of hormone release (Wakerley *et al.*, 1978).

The activation of voltage-dependent Ca^{2+} channels on the plasma membrane of the MNCs is critical for exocytosis, but these channels do not act in isolation. There are a plethora of channels in the MNCs that influence the membrane potential and therefore the response of these channels. Potassium currents contribute significantly to pattern generation (Roper *et al.*, 2003). Loose-patch clamp recordings done on the terminals and somata of the MNCs *in situ* confirm the presence of Na^+, "A"-type K^+ and Ca^{2+}-activated K^+ as well as voltage-dependent Ca^{2+} currents in both locations (Marrero & Lemos, 2003). The Kv3-like high-threshold K^+ currents observed in the SON in slice preparations and acutely isolated neurons may be important in maintaining spike width and Ca^{2+} influx during repetitive firing (Shevchenko *et al.*, 2004). Immunochemical studies revealed a stronger expression of the Kv3.1b alpha-subunit in VP neurons, which may be related to the greater importance of this current type in VP spike repolarization (Shevchenko *et al.*, 2004). As discussed above, the stretch-inactivated cation channels also modify membrane potential.

There are many receptors on the plasma membrane of MNC somata, and the binding of the appropriate ligand will have an effect on the cell function. Some of these receptors are estrogen β (Sladek & Somponpun, 2004), ionotropic glutamate (Tasker *et al.*, 2002), muscarinic cholinergic (Ghamari-Langroudi & Bourque, 2004), histamine H1 (Smith & Armstrong, 1993), κ-opioid

(Brown & Leng, 2000), vasopressin V_{1a} and V_{1b} (Hurbin *et al.*, 2002), and oxytocin (Adan *et al.*, 1995). The presence of the latter two implies the potential for autocrine control through somatodendritic release of the hormone. Furthermore, the excitatory glutamatergic inputs derived from forebrain regions (Bourque, 1998) influence the rate of AP discharge of MNC somata, which will in turn determine the rate of hormone release from the NH.

Ultrastructural plasticity

Extensive structural remodeling occurs within the SON during times when there is an increased physiological need for VP and/or OT (Tasker *et al.*, 2002). These changes include somatic hypertrophy, decreased glial coverage and increased synaptic innervation. It was thought that these changes, regardless of the stimulus, were the exclusive domain of the OTergic cells (Chapman *et al.*, 1986; Theodosis *et al.*, 1986). However, it is now known that the VPergic neurons are also involved, especially during periods of water deprivation (Oliet, 2002). The remodeling is not limited to the somata but pervades all levels of the hypothalamic neurohypophysial system (HNS) including the dendrites and terminals, where the changes mirror the altered functional properties of the system (Hatton, 1997).

The glial cells are required for uptake of neurotransmitters including glutamate, and the impairment of glutamate clearance in the SON of lactating and chronically dehydrated animals results in enhanced activation of presynaptic metabotropic glutamate receptors that inhibit transmitter release (Oliet & Piet, 2004). Other consequences of glial retraction include: decreased spatial buffering of K^+ contributing to neuronal depolarization; decreased taurine release from astrocytes, which has an inhibitory effect on the MNCs (Hussy, 2002); and enhanced autostimulatory somatodendritic release of the OT and VP from the MNCs (Hatton, 1997). All of these modifications lead to an enhancement of neuronal excitability.

Under control conditions, the fine astrocytic processes separating the somata and afferent synapses are sparse with individual boutons contacting only one postsynaptic element. The remodeling that occurs in parturition and lactation results in double synapses and the formation of dendritic bundles (Perlmutter *et al.*, 1984), and this enhanced dendodendritic contact may enhance the pattern generating network characteristic of the OT neurons (Leng *et al.*, 1999). Remodeling resulting from water deprivation includes increases in the percentage of somatosomatic apposition and rehydration reverses these changes (Theodosis & Poulain, 1993).

Within the NH, physiological stimulation causes reversible pituicyte retraction providing increased abutment of the terminals with the vascular beds rather than enlargement or sprouting of magnocellular terminals themselves (Miyata *et al.*, 2001). The VP receptor V_1R is present in rat pituicytes, where it is implicated in the modulatory role that allows high output of both VP and OT. In the resting state, ultrastructural evidence shows that the pituicytes act as a barrier between terminals and epithelium of vascular beds. Rosso *et al.* (2004) proposed that those stressors that stimulate the release of the VP also cause the pituicytes to retract from around the blood vessels. In this way, the terminals have increased epithelial contact and therefore more surface onto which the granules containing the peptide can fuse and release their contents into the capillary bed for systemic distribution.

Supporting a physiological requirement for ultrastructural plasticity, many molecules that are concerned with dynamic structural remodeling are highly expressed in HNS but generally at low expression levels in other regions of adult brains. These include: cytoskeletal proteins such as tubulin, microtubule-associated proteins, and intermediate filaments proteins; and cell adhesion molecules (immunoglobulins and extracellular matrix glycoproteins) that are known to

participate in neuronal-glial, neuronal-neuronal and glial-glial recognition and guidance (Miyata & Hatton, 2002).

Morphologically reversible ultrastructural modifications in the SON (Theodosis *et al.*, 1995) and NH (Miyata *et al.*, 2001) do occur and are important in the physiological functioning of the cells. Various inputs are altered and the excitability of the cell is affected, and the extent to which the properties of the MNC are impacted is not yet fully understood.

The secretory granule

Vasopressin and oxytocin are the primary peptides synthesized and released from the somata of the MNCs. There are four phases in the secretion of neurosecretory products: synthesis of the molecules; packaging into granules; transport to the sites of release; and release into the extracellular space. The nontranslated introns are removed prior to the movement of the mRNA out of the nucleus and into the cytoplasm. Processing of the preprohormone occurs in the cisternae of the rough endoplasmic reticulum (RER) as the dilated RER has been identified as the site of active protein synthesis (Brownstein *et al.*, 1980). The preprohormone consists of the signal prepeptide, a hormone peptide and a prepeptide neurophysin-copeptin. Before the molecule leaves the RER, the signal peptide is removed, initial glycosylation of VP occurs, and the disulfide bonds between cysteine residues enables the primordial folding. The modified precursor is then translocated to the Golgi complex where the next phase of processing takes place, the packaging of the prohormone into the membrane-bound secretory granules. The prohormones leave the trans-Golgi network via the regulated secretory granules (Eaton *et al.*, 2000). ATPase in the granule membrane generates a pH gradient and the acidic environment required by the processing enzymes expressed within the granules. Successive processing including glycosylation, proteolysis and amidation, occurs in distinct compartments and this results in domain separation and reshaping. An enzymatic cascade, which includes a Lys-Arg

Ca^{2+}-dependent endopeptidase, a carboxypeptidase E-like enzyme, a peptidylglycine α-hydrosylating monooxygenase and peptidyl-α-hydroxyglycine α-amidating enzyme, acts upon the hormone before it becomes bioactive (Acher *et al.*, 2002).

Normally, the constitutive pathway brings newly synthesized proteins and hormones to the plasma membrane in transport vesicles in a non-regulated, maintenance fashion. A basal level of VP and OT can be maintained through this mechanism. However, in the MNCs, the constitutive pathway is augmented by regulated transport to deliver the hormones for secretion and this pathway involves the assembly and aggregation of the hormones into dense core granules. Aggregation and assembly into oligomers with a membrane-associated form of the regulated secretory protein occurs in the lumen of the trans-Golgi network. The nascent bud includes the soluble NSF attachment protein receptors (SNARE) molecules, soluble non-secretory proteins, and membrane proteins. Aggregation into dense core granules allows the secreted hormones to be packaged at concentrations up to 200 times higher than the unaggregated protein in the Golgi lumen. This enables secretory cells to release large amounts of the protein promptly when triggered to do so.

A number of other peptides are also synthesized by the VP and OT cells, including dynorphin, an endogenous opioid that is co-packaged with VP (Whitnall *et al.*, 1983), and galanin (Landry *et al.*, 2003). The latter is especially interesting in that mRNA for galanin and VP were detected at different domains of the ER, suggesting that there is partial segregation of the two secretory granule populations, allowing preferential targeting towards dendrites or terminals where they could have different functions, autocrine/paracrine and endocrine, respectively. At least two types of channels are present in neurosecretory granule membranes, a small conductance (~30 pS) channel, and a large, multiple-conductance (62-232 pS), Ca^{2+}-regulated cation channel that

has similarities to the vesicular membrane protein synaptophysin. The latter appears to be directly involved in the mechanism of peptide release from the terminals (Yin *et al.*, 2002). The membrane also contains those proteins required for the docking of the granule with the exocytotic complex.

Axonal transport is required for the granules to be delivered to the terminals. In the neurohypophysial system, the four rate components of axonal transport have been demonstrated *in vivo*. These are: fast anterograde and fast retrograde transport of membrane-bound organelles, at 50-400 mm/day; slow component b containing microfilament, clathrin, and general metabolic proteins, at 2-8 mm/day; and slow component a containing microtubule and neurofilament proteins required for the cytoskeleton, at 0.2-1.0 mm/day (Gainer & Chin, 1998). The large dense core vesicles (LDCV) are transported at 10 mm/hr (Hille, 2001) and are therefore in the fast component. Fast axonal transport uses ATP-powered kinesin motors to drag the granules along tracks of axonal microtubules.Treatment with colchicine, which is known to prevent microtubule extension, inhibits transport. The granules are transported in clusters along the bundle of parallel microtubules that form a track within the axon, and this is seen in the beaded appearance of the axons. It takes 1-2 hours for the entire process of synthesis, transport and storage in the NH (Kam *et al.*, 2004).

Once the granules reach the NH, they are either stored in Herring bodies that are generally positioned away from the basal lamina or go directly to the plasma membrane. There appear to be two distinct pools of the granules, those available for immediate release, and those in storage (Giovannucci & Stuenkel, 1997). The mechanism of this apparent differential sorting is not well understood although one could assume that the stimulus for release could play a role. There are a number of potential targets including phosphorylation of the SNARE proteins on the granule or

another granule membrane protein, or perhaps there is a modification of the microtubule orientation or track. There needs to be some sort of recognition sequence on the granule that allows it to respond appropriately to the stimulus. However, the perturbations that induce release require an immediate response, and therefore hormones released must already be at the plasma membrane awaiting a signal to go, or targeted there directly. It would be physiologically inefficient for granules to be synthesized and placed in storage at times of immediate need.

It is estimated that only 5-10% of the stored VP is turned over daily, through release and degradation. The large storage pool of VP in the NH is sufficient to maintain VP release for 30 to 50 days under basal conditions and 5-7 days at maximal release rates (Sladek, 2000). This storage pool appears to be built up during development when VP synthesis exceeds VP release and is maintained thereafter in the mature animal by matching synthesis to release. However, when chronic dehydration is followed by a period of rehydration, VP mRNA content is elevated for a period of several weeks. The extension of this period of excessive synthesis allows replenishment of the hormone stores, and once this is complete, VP mRNA returns to basal levels (Zingg *et al.*, 1986). Therefore, the events following dehydration include a period of increased synthetic activity in the MNC somata.

Somatodendritic release

Regulated central release of the hormones from the MNCs has been well established through microdialysis studies that measure OT and VP in the extracellular space (Moos *et al.*, 1998), capacitance measurements that confirmed Ca^{2+}-dependent exocytosis (Soldo *et al.*, 2004), and with ultrastructural visualization using tannic acid to capture exocytosis (Pow & Morris, 1989). Not only are the dendrites capable of releasing hormone, they also appear to have the cellular components necessary to synthesize proteins for local release (Morris *et al.*, 2000). Therefore, central release appears to be an important modulator of cellular activity, and it does not act in

parallel with axonal release. There are several possible explanations for this distinction, including: the independent peptide biosynthesis and storage of neurosecretory granules in dendrites; compartmentalization of receptor populations and/or second-messenger systems between dendrite and soma; mediation of changes in intracellular Ca^{2+} via second-messenger systems in dendrites and by voltage-dependent channels in the soma; and dissociation of electrical activity in axons and dendrites (Ludwig *et al.*, 2002). Antidromic activation of electrical activity in the cell bodies releases VP from dendrites only after the cytosolic Ca^{2+} concentration is increased by thapsigargin pretreatment (Ludwig *et al.*, 2005). Thapsigargin binds to the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps and inhibits Ca^{2+} reuptake, thereby increasing cytosolic Ca^{2+} concentrations. VP itself is effective in inducing dendritic VP release, but it is ineffective in preparing the cell for release (priming) (Ludwig *et al.*, 2005). This priming appears to translocate the dense-core granules closer to the dendritic membrane (Tobin *et al.*, 2004).

The cell bodies of the MNCs contain receptors for both OT and VP, suggesting a role for central neuropeptide release on function. The OT binding sites are concentrated on OTergic cell bodies and dendrites, but not on their axons and endings in the NH. The effect of OT binding in the SON is mediated by endocannabinoids, with OT playing an important role in endocannabinoid signaling (Hirasawa *et al.*, 2004). This implies that somatodendritic peptide release and action on previously identified autoreceptors facilitates the release of endocannabinoids that act as mediators of presynaptic inhibition (Hirasawa *et al.*, 2004). Somatodendritic release of VP may contribute to the optimization of the neuronal discharge according to the physiological demand (Gouzenes *et al.*, 1998). Two VP receptors, V_{1a} and V_{1b}, are located in VPergic somata and colocalized with VP in cytoplasmic vesicles. These G_q-protein

coupled receptors acting through phospholipase C, catalyse the hydrolysis of phosphatidylinositol 4,5-bisphosphate to form two active subunits, inositol trisphosphate (IP_3) and diacylglycerol. The IP_3 mobilizes Ca^{2+} from the endoplasmic reticulum. V_{1a}-receptor mRNA levels are upregulated with dehydration, and their colocalization might explain how V_{1a} autoreceptors are controlled by cell activity and/or local concentrations of VP released, which allows fine adjustments in MNC electrical activity (Hurbin *et al.*, 2002). The synchronous and pulsatile firing pattern of the stimulated VPergic somata responsible for the efficient delivery of systemic hormone may rely on the interplay of the signaling pathways that the VP receptors initiate and this could contribute to pattern generation (Hurbin *et al.*, 1998).

There are several implications of the local release of OT and VP, which include: autoregulation through modulation of release of excitatory amino acids from afferent terminals targeting these cells and/or from other cellular sources (Curras-Collazo *et al.*, 2003); control of the interactions between OTergic neurons and their surrounding glia; and facilitation or inhibition of the electrical activation of the neurons (Morris & Ludwig, 2004). Somatodendritic release of OT regulates the OTergic neurons during lactation by reducing inhibitory synaptic transmission to these neurons. In lactation, there are increases in the number of OT vesicles. The result of this is increased inhibition of GABAergic synaptic transmission (De Kock *et al.*, 2003) and augmentation of noradrenaline release through a pre-synaptic action during the milk ejection reflex (Onaka *et al.*, 2003). There also appears to be a developmental requirement of somatodendritic release where the self-sustaining positive feedback loop is maximal during the second postnatal week and decreases thereafter (Chevaleyre *et al.*, 2000), implicating it in neuronal maturation.

The dense-core granules released by the MNCs also contain much lower concentrations of other neuroactive peptides including cholecystokinin (CCK), thyrotropin releasing hormone (TRH), galanin, dynorphin and enkephalin, the latter two likely acting on local pre- or post-synaptic opioid receptors. Since dendritic VP release is increased and prolonged after various stimuli, this mechanism may act to restrain excitation of VP neurons (and hence VP secretion from the NH) during continuing stimulation (Ludwig & Leng, 1998).

Ca^{2+} Channels

Voltage gated Ca^{2+} channels

Calcium ions play an integral role in a wide array of cellular functions, including secretion, muscle contraction, gene transcription and cell proliferation. Therefore, the movement and cytosolic concentration of Ca^{2+} must be tightly regulated, especially since excessive calcium is toxic to the cell. There are a number of ways for the ion to move into and out of the cytosol, including pumps, transporters and voltage-dependent Ca^{2+} channels (VDCC). The latter are membrane-spanning proteins with a calcium-selective pore, whose opening and closing is controlled by voltage. VDCC are present in neurons, muscle and endocrine cells, all of which are excitable cells, but also in nonexcitable cells such as osteoblasts (Meszaros *et al.*, 1996). The primary function of the VDCC in excitable cells is to transduce an electrical signal, the action potential, into an intracellular signal, the rise in intracellular Ca^{2+}. The secondary function is to further depolarize the membrane potential through the entry of the positively charged ion. The calcium currents produced by the VDCC are referred to as L-, N-, P/Q-, R- and T-type currents and have been characterized electrophysiologically (Catterall, 2000).

As illustrated in Figure 1.2, the VDCC consists of four subunits, α_1-, β-, α_2-δ- and γ-subunits. The largest constituent, with a molecular mass of 190-250 kDa, is the membrane spanning, voltage-sensing conduction pore-forming α_1-subunit and it is this subunit that determines most

of the channel properties. Coded on a single gene, the 24 α-helical transmembrane segments are organized into four repeated domains (I-IV) of 6 segments (S1 to S6) (Tanabe *et al.*, 1987). Positively charged amino acid residues in the S4 region of each domain confer the voltage dependence (Jones, 2003), while the ion-selectivity is conferred by the P-region, the loop between the fifth and sixth transmembrane segment of each domain. To date, 10 α_1-subunits have been identified and cloned, as well as numerous isoforms, resulting from alternative splicing (Bourinet *et al.*, 1999; Ghasemzadeh *et al.*, 1999; Pan & Lipscombe, 2000; Safa *et al.*, 2001; Lipscombe *et al.*, 2002; Lin *et al.*, 2004; Tang *et al.*, 2004).

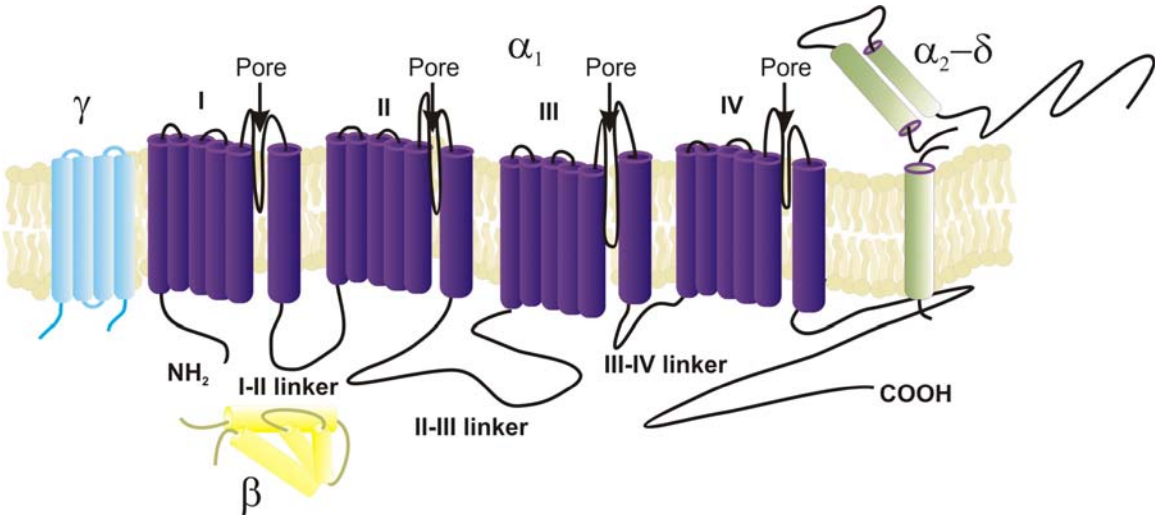

Figure 1.2. Structure of the voltage-dependent Ca^{2+} channel**.** The generic voltage-gated Ca^{2+} channel consists of four subunits. The defining subunit is a membrane-spanning, pore-forming, voltage-sensing α_1-subunit (purple). There is a cytosolic β-subunit (yellow) and it plays a role in membrane trafficking and targeting of the α_1-subunit to the plasma membrane. The two other subunits are α_2-δ (green) and γ (blue), the latter only recently isolated in neuronal preparations.

Based on the properties attributable to the α_1-subunit, VDCC have been divided into three families, Cav1, Cav2 and Cav3 (Ertel *et al.*, 2000). Table 1.1 summarizes the various subtypes and their distinctive characteristics and locations. The L-type channels make up the Cav1 family and the first channel cloned, Cav1.1 or α_{1S}, is associated with skeletal muscle cells where it is

responsible for the initiation of excitation-contraction coupling. Cav1.2 and Cav1.3, also called α_{1C} and α_{1D}, are found in the brain where they are involved in endocrine secretion and gene transcription (Bading *et al.*, 1993). The L-type Ca^{2+} channels are regulated primarily by second messenger-activated protein phosphorylation pathways (Catterall, 2000). In general, these channels produce long-lasting or noninactivating current and large single-channel currents. This family of channels is sensitive to dihydropyridines (DHP), such as nifedipine, nitrendipine, and isradipine. This trait has been used clinically in the treatment of hypertension. The reduced entry of calcium into the arteriolar smooth-muscle cells results in the relaxation of the blood vessel with a subsequent decrease in tension.

Table 1.1. Ca^{2+} channel diversity

Name		Channel Subtype	α_1-subunit	Location	Functions
Cav1	1.1	**L**	α_{1S}	Skeletal muscle	Excitation-contraction coupling, Ca^{2+} homeostasis
	1.2		**α_{1C}**	Cardiac muscle, endocrine cells, neurons	Endocrine and neuroendocrine secretion, slow release from neuronal terminals and dendrites, graded potential synapses, gene regulation
	1.3		**α_{1D}**	Endocrine cells, neurons, cochlea	
Cav2	**2.1**	**P/Q**	**α_{1A}**	Nerve terminals, dendrites, cochlea	Neurotransmission, endocrine and neuroendocrine secretion, dendritic Ca^{2+} transients
	2.2	**N**	**α_{1B}**	Nerve terminals, dendrites	
	2.3	R	α_{1E}	Cell bodies, dendrites, nerve terminals	
Cav3	3.1	T	α_{1G}	Cardiac muscle, skeletal muscle, neurons, retina, cochlea	Helping generate spontaneous or pacemaking electrical activity
	3.2		α_{1H}	Cardiac muscle, neurons, kidney, liver	
	3.3		α_{1I}	Neurons	

The channels in bold are thought to be the most important in the MNCs (Adapted from Ertel *et al.*, 2000; Fisher & Bourque, 2001).

The Cav2 family is made up of Cav2.1 (α_{1A}), Cav2.2 (α_{1B}) and Cav2.3 (α_{1E}) or P/Q-, N- and R-type channels respectively. The P/Q- and N-type channels are known to initiate rapid synaptic transmission, and are regulated primarily by direct interaction with SNARE- (Sheng *et al.*, 1998) and G-proteins (Dolphin, 1998) and secondarily by protein phosphorylation (Catterall, 1999).

The Cav2.1 currents are sensitive to the funnel spider venom, ω-agatoxin-IVA (Mintz *et al.*, 1992) and to the marine snail toxin ω-conotoxin-MVIIC (Hillyard *et al.*, 1992), while the Cav2.2 current is blocked by the marine snail toxin ω-conotoxin-GVIA (Olivera *et al.*, 1984). The spider toxin SNX-482 has been shown to be selectively antagonistic to the Cav2.3 channel, although total blockage of the current has not been shown (Sochivko *et al.*, 2002). The final voltage-dependent current that makes up the Cav3 family is the T-type. These low-voltage activated currents are activated and inactivated more rapidly and at more negative membrane potentials than other Ca^{2+} currents and control action potential burst generation and hormone secretion (Wolfe *et al.*, 2003).

At a resting potential of -70 mV, the Cav1 and Cav2.2 channels are closed. They are maximally opened by depolarization to +10 mV, which occurs during the depolarization generated by an action potential in muscle cells and neurons. The Cav3 channels require a much smaller depolarization and therefore will open at voltages near the resting potential, enabling the positively charged calcium ion to enter and thus depolarize the membrane potential, which can help generate spontaneous electrical activity.

The β-subunit is a small (50-80 kDa), intracellular protein that interacts with the cytoplasmic linker of the I-II loop of the α_1 subunit (Scott *et al.*, 1996). Its functional role is targeting of the channels (Chien *et al.*, 1995; Namkung *et al.*, 1998; Brice & Dolphin, 1999; Gao *et al.*, 1999; Walker *et al.*, 1999) and altering the biophysical properties of the channel (De Waard & Campbell, 1995; Gerster *et al.*, 1999; Smith *et al.*, 1999). The β-subunits may also play a role in the transformation of Cav2 channel function in immature neurons and mature synapses (Spafford *et al.*, 2004). Less is known about the α_2-δ-subunit, composed of a pair of proteins linked by a disulfide bridge, with a single transmembrane segment. It is thought that it plays a role in the

regulation of intracellular processing and enhances channel expression (Arikkath & Campbell, 2003). In concert with various β-subunits, it can modify channel properties, including the affinity of the channel for specific toxins (De Waard & Campbell, 1995). Even less well understood is the transmembrane γ-subunit, first identified in skeletal muscle, but also found in neurons. Eight γ-subunits have been cloned to date, with γ_1 restricted to skeletal muscle, whereas the other seven have all been found in brain (Arikkath & Campbell, 2003). *In vivo*, the γ-isoforms form complexes with VDCC and AMPA receptors, and are differentially and selectively expressed in neuronal processes (Sharp *et al.*, 2001).

Ca^{2+} channels in MNCs

Expression studies in cell lines have allowed researchers to study Ca^{2+} channel targeting (Brice & Dolphin, 1999) and effects of co-expressed auxillary subunits (Walker *et al.*, 1999; Dubel *et al.*, 2004) and this knowledge has greatly enhanced our understanding of the function of the channel *in vivo*. However, the artificial and necessarily simple expression system models do not necessarily reflect what is actually happening *in situ*. It is in this context that the study of the Ca^{2+} channels in the MNCs provides the opportunity to investigate the distribution of the channels within compartments of the same cell and determine whether this distribution is correlated to different functions.

The presence of the various voltage-activated calcium channels in the MNCs has been demonstrated using RT-PCR (Glasgow *et al.*, 1999). These researchers found that the subunit genes, $\alpha_{1A\text{-}D}$, but not α_{1E}, are expressed in virtually all MNCs. These findings confirm earlier electrophysiological studies which showed that the Ca^{2+} currents of the isolated somata (Fisher & Bourque, 1995; Foehring & Armstrong, 1996) and terminals (Fisher & Bourque, 1996) are composed of a variety of VDCC. Electrophysiologically, the N-, P/Q- L- and R-type but not T-

type currents were identified by Joux *et al.* (2001). However, a previous study by Fisher and Bourque (1995) found that the MNC somata did express a T-type current, along with N-, L- and P-types and a low threshold nifedipine-sensitive current. The latter is likely through the α_{1D} subtype of the L-type current-generating Ca^{2+} channels as it has a lower threshold than α_{1C}.

Based on the patch-clamp studies of the Ca^{2+} currents, the predominant channel is the N-type, followed by P/Q- and L-type.There are differences in the current properties within compartments suggesting segregation and that current types defined pharmacologically may have different kinetic properties (Fisher & Bourque, 1996). The presence of the α_{1A-D}-subunits in tissue homogenates from the SON and the NH (Fisher *et al.*, 2000) was confirmed in the lab using Western blot analysis (data not shown).

Immunofluorescence studies on isolated NH terminals found punctate staining for several of the α_1-subunits within the intracellular space, suggesting that there is an internal reservoir of the channels in the terminals (Fisher *et al.*, 2000) that could be associated with the neurosecretory granules. The N-type Ca^{2+} channels have been observed on the microvesicles but not the granules from the neurosecretory nerve terminals of the NH (Zhang *et al.*, 2000). The presence of the channel on either the granules or microvesicles raises an interesting possibility; that the channel can be translocated to the plasma membrane in a regulated fashion during times of increased hormone release. There are several examples of the effectiveness of translocation for bringing channels to the plasma membrane, including the downstream effect of VP during dehydration. Systemically released VP binds with high affinity to the V_2 receptor (V_2R). This G_s-protein coupled receptor is found in high density on the basal surface of epithelial cells of the distal convoluted tubules and collecting ducts of the renal medulla. When VP binds, receptor mediated endocytosis occurs and adenylate cyclase type IV is activated. Intracellular cAMP is

increased and protein kinase A phosphorylates the aquaporin2 (AQP_2) protein. This specific water channel moves from intracellular vesicles via cytoskeleton microtubular and microfilamentous elements to the luminal membrane. The channel is inserted when the vesicle and the plasma membrane fuse and water moves along an osmotic gradient from the hypotonic tubular urine to the hypertonic interstitial fluid of the renal medulla. The inserted water channels therefore control the amount of water that is reabsorbed. Once osmolality is restored to the normal range, neurohypophysial VP secretion returns to constitutive levels. The AVP-V_2R is degraded rather than recycled (Robben *et al.*, 2004), and the luminal membrane is once more impermeable. The net water-sparing effect is concentration of the urine to maintain fluid homeostasis. Bag cell neurons of *Aplysia californica* have an internal store of Ca^{2+} channels and these are translocated to the plasma membrane during the egg laying process (White & Kaczmarek, 1997). An intracellular pool of N-type Ca^{2+} channels has been described in different neuronal cell lines, including IMR32 human neuroblastoma and PC12 rat pheochromocytoma cells, and these channels are translocated to the plasma membrane when the cells are exposed to secretagogues such as high K^+ (Passafaro *et al.*, 1996).

Study Rationale

Ca^{2+}-dependent currents are critical to the regulation of the firing patterns that potentiate hormone release from the terminals of the MNCs. These firing patterns are markedly changed by dehydration or parturition/lactation. These changes coincide with the ultrastructural reorganization of the SON and NH that is thought to lead to enhancement of secretion (Theodosis *et al.*, 2004). De Kock *et al.* (2003) reported an upregulation of Ca^{2+} currents in the OTergic somata of lactating rats, while Teruyama & Armstrong (2005) report that although Ca^{2+} current was slightly larger on OTergic cells during lactation, current density was unchanged when corrected for somatic hypertrophy.

Changes in the number or properties of the Ca^{2+}-conducting channels could have significant consequences on the functioning of the MNCs, as well as other secretory cells. In addition to long and short term changes in the firing patterns of the cells, other possible physiological effects could be to increase gene transcription and/or increase hormone release from the somatodendritic regions and terminals. Translocation of Ca^{2+} channels from internal stores to the plasma membrane occurs in other systems, including the bag cell neurons of *Aplysia californica* (White & Kaczmarek, 1997) and sea urchin eggs at fertilization (Smith *et al.*, 2000). This mechanism could also be employed by the MNCs as a means of modifying the Ca^{2+} currents during dehydration and lactation/parturition.

In light of the ultrastructural and electrophysiological changes observed in the MNCs following stimulation, and the presence of intracellular stores of Ca^{2+} channels, the purpose of this study was to determine whether specific Ca^{2+} channel subtype density is changed following a period of water deprivation.

Hypothesis and objectives

Hypothesis

This study tests the hypothesis that the density of Ca^{2+} channels in the MNCs within the SON and NH is modified following a period of water deprivation.

Objectives

The first objective of the study was to quantify the density for each of the major Ca^{2+} channels in the SON and NH and to compare this to the channel density following a period of water deprivation. To address this, radioligand binding studies with ligands specific to the N-type, P/Q-type and L-type α_1-subunit were done.

The second objective was to determine if any change observed was the result of *de novo* synthesis of channels. The mRNA levels in the SON from control and dehydrated animals were compared using RT-PCR and primers for each of the α_1-subunits.

The third objective was to visualize the localization of specific channels and determine whether these channels were differentially distributed following the period of water deprivation. Immunocytochemical studies were done using antibodies specific to the α_1-subunits.

CHAPTER 2
MATERIALS AND METHODS

The rats

Male Long Evans rats (Charles River, QC) weighing 225-250 g, were housed in pairs with *ad libitum* access to rodent chow and water, with at 12 hour light/dark cycle. In order to induce a dehydrated state, the water bottle was removed from the cage for a period of 16 or 24 hours prior to sacrifice. The radioimmunoassays were done initially following the 16-hour regime as this length of time is known to increase plasma osmolality maximally (Wakerley *et al.*, 1978) and induce ultrastructural modifications (Tweedle & Hatton, 1977). This period was extended to 24 hours for the RT-PCR studies in order to amplify the effect of dehydration of mRNA. Further binding studies were done using the 24-hour protocol and these results were not different from those acquired from the 16-hour protocol.

Isolation of MNC somata and terminals

At 7-11 weeks of age, rats were anaesthetized by halothane inhalation and decapitated using a small rodent guillotine, in accordance to the University of Saskatchewan Animal Care Protocol UCACS#20000121. The head was immediately placed in ice-cold physiological saline. The scalp was opened using scissors to expose the skullcap, and the cerebellum and attached spinal column were detached. Rongeurs were inserted just below the bone and the skullcap lifted to expose the cerebrum. The brain was carefully lifted within the cranial vault, the optic nerves cut distal to the cerebrum, and the dissociated brain placed with the inferior surface exposed. A drop of homogenization buffer was applied to both the brain and the pituitary, which remained in the cranial cavity. The pituitary was then removed and placed in a dissecting dish containing

homogenization buffer (on ice), and under the dissecting microscope, the NH containing the MNC terminals, was detached from the adenohypophysis and intermediate lobe.

To isolate the SON, two coronal cuts were made with a razor blade, the first immediately anterior to the optic chiasma and the second, parallel cut at 1.0 mm. The tissue slice containing the SON was placed in buffer and secured with pins. Under the dissecting microscope, bilateral cuts distal to the SON were made and the membrane gently removed. Cuts were made at ~45° to the plane of the section on the lateral face of each SON, and a second cut medially. A bilateral third cut to connect the first two produced two tissue segments containing a portion of the SON. (See Figure 2.1)

Tissue Preparation

Rat Brain. The initial saturation-binding curve was done using whole brain tissue, which ensured a large amount of neuronal protein. Approximately 1/4 of the rat brain was placed in 2ml homogenization buffer (HB). The tissue was finely diced using scissors, transferred to a 10 ml hand Dounce homogenizer containing an additional 3 ml HB, and homogenized with the PolyTron. The homogenate was then transferred to a plastic centrifuge tube and centrifuged at 17000 rpm for 15 minutes, at 4ºC. The supernatant was discarded and the pellet was resuspended in 3 ml HB. The BioRad Protein Assay was done to determine the protein content of the samples based on a bovine serum albumin (BSA) standard curve.

Supraoptic nucleus. The enzymatic isolation of the MNC somata of the SON was done as previously described (Oliet & Bourque, 1992) with modifications. The osmolality of the buffer was checked using a Vapro® vapour pressure osmometer (Wescor); solutions were 295 mOsmol/kg for control and 320 mOsmol/kg for tissue from water-deprived animals. Buffers

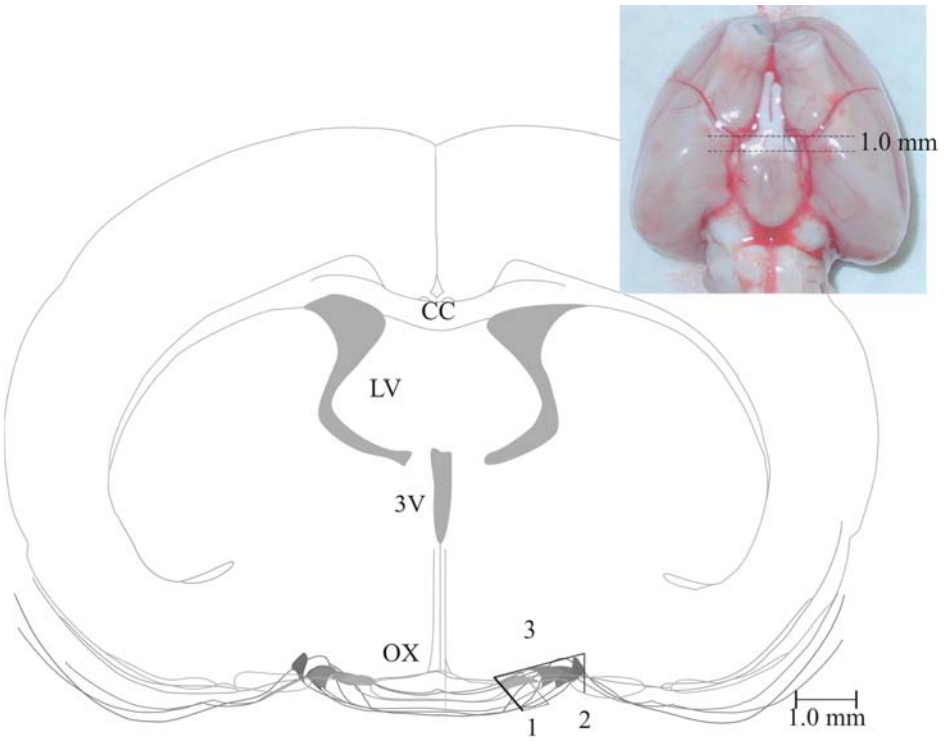

Figure 2.1. Isolation of the SON. This diagram represents the surgical isolation of the SON, and was generated by overlaying coronal slices from the Rat Brain Atlas. All distances refer to Bregma. The top, full view (lightest grey) is -0.80 mm and is what is seen with the slice under the microscope. Landmarks are the lateral ventricles (LV), third ventricle (3V) corpus callosum (CC), and optic chiasma (OX). As the image moves down through the slice, to -0.92, -1.40 and -1.80, the SON becomes darker. Insert is a photograph of the dorsal surface of the brain and the lines indicate the location of the 1 mm slice used. Following removal of the membrane, the first cut (1) is made at an angle of ~45°, parallel to the OX, the second (2) is made at the cleft, and the third (3) connects the first two cuts. A piece of tissue measuring approximately $1mm^3$ containing the majority of the MNC somata within the SON is isolated.

were modified with the addition of mannitol. The SON were placed in a 2 ml Dounce hand homogenizer containing 500 µl HB and kept on ice. The tissue was manually homogenized until the buffer appeared cloudy and no distinct tissue pieces were visible. The SON were collected from 4 rats and each nuclei pair were homogenized immediately following isolation to minimize degradation. Additional HB was added to make a final volume of 600 µl. The BioRad Protein Assay was done to determine the protein content of the sample.

Neurohypophysis. Following the isolation of the NH, it was placed in the 2 ml Dounce hand homogenizer containing 500 µl HB of the appropriate osmolality, and kept on ice. The tissue was manually homogenized until the buffer appeared cloudy and no distinct tissue pieces were visible. The NH from 4 rats within a treatment group were pooled in order to ensure adequate protein for the radioligand studies. Each NH was homogenized immediately following isolation to minimize degradation. A final volume of 600 µl was ensured with additional HB. The BioRad Protein Assay was done to determine the protein content of the sample.

Radioligand binding studies

^{125}I-ω-Ctx-GVIA binding to N-type Ca^{2+} channels

Iodinated ligands have a half-life of 59 days and the energy released at the time of decay is catastrophic. This explosive reaction produces molecular biproducts of undefined constitution that could potentially interfere with the binding studies. It is imperative that experiments be carried out as soon as possible following the iodination of the ligand; therefore, each shipment of ^{125}I-ω-Ctx-GVIA was used within one week of arrival to minimize contamination and standardize results.

SON and NH tissues were prepared as described above with the following modifications: binding was carried out in HB Buffer A (Hepes 10 mM, BSA 0.02%, NaCl 100 mM, pH 7.3) with glucose (25mM), one Complete Mini protease inhibitor tablet (Roche) and pepstatin (1.5 µM). Saturation binding curves were generated from 0.005 to 0.6 nM ^{125}I-ω-Ctx-GVIA (PerkinElmer) in a total volume of 200 µl (5-10 µg protein/assay) with non-specific binding determined following 30 minute preincubation at room temperature with the non-iodinated ω-Ctx-GVIA (0.1 µM) (Alomone Labs). The specific activity of the ligand was decreased from 2200 to 550 Ci/mmol by dilution 1:3 in ω-Ctx-GVIA at the same concentration. Following a 90

minute incubation at room temperature, the tubes containing the samples were placed in ice water to stop the reaction. They were then vacuum-filtered through Whatman GF/A Glass fibre filters that had been pre-soaked in 0.3% polyethyleneimine, washed 3 times with Buffer A, and placed in glass counting tubes. The filters were read immediately on the LKB Wallac 1271 Riagamma Automatic Gamma Counter. Effective concentration of the ^{125}I-ω-Ctx-GVIA was determined from a standard curve generated from known volumes of the ligand. B_{MAX} was determined from the binding curve of the difference between total and non-specific binding using GraphPad Prism3 software.

^{125}I-ω-Ctx-MVIIC binding to P/Q-type Ca^{2+} channels

SON and NH tissues were prepared as described above with the following modifications: binding was carried out in HB Buffer A with EDTA (100 nM) and EGTA (100 nM). The saturation binding curve was generated from 2.5 pM to 1.0 nM of ^{125}I-ω-Ctx-MVIIC (PerkinElmer) in a total volume of 200 µl (5-10 µg protein/assay) with non-specific binding determined following 30 minute preincubation at room temperature with the non-iodinated ω-Ctx-MVIIC (0.1 µM) (Alomone Labs). This was supplemented with 1 µM ω-Ctx-GVIA in order to decrease binding of ω-Ctx-MVIIC to N-type channels. The specific activity of the ligand was decreased from 2200 to 440 Ci/mmol by dilution 1:4 with ω-Ctx-MVIIC at the same concentration. Following 90 minutes incubation at room temperature, the tubes containing the samples were placed in ice water to stop the reaction. They were then vacuum-filtered as above, washed 3 times with Buffer A, and placed in glass counting tubes. The filters were read immediately on the LKB Wallac 1271 Riagamma Automatic Gamma Counter. The effective concentration of the ^{125}I-ω-Ctx-MVIIC was determined from a standard curve generated from

known volumes. The B_{MAX} was determined from the binding curve of the difference between total and non-specific binding using GraphPad Prism3 software.

^{3}H-isradipine binding to L-type Ca^{2+} channels

The specific activity of the ^{3}H-isradipine is very low at 86 Ci/mmol and a detectable signal requires a large amount of protein. The SON and NH yield only a small amount of protein and a full saturation binding curve was not possible using these tissues. Therefore whole rat brain samples were prepared as described above with the following modifications: binding was carried out in HB consisting of Tris-HCl 50 mM, glucose 25 mM, pepstatin 1.4 μM, $CaCl_2$ 1 mM, and one Complete Mini protease inhibitor tablet (Roche). For the full saturation-binding curve, the total volume of each assay was 1000 μl with total protein ~80 μg and concentrations of the ^{3}H-isradipine ranging from 0.05 to 1.6 nM. Glass culture tubes containing the samples were incubated for 90 minutes under minimal light conditions. Nonspecific binding was determined by preincubating samples for 30 minutes with 1.5 μM nifedipine (in 0.15% DMSO). Following the incubation, tubes were placed on ice in order to stop the reaction in all tubes at the same endpoint. Samples were then vacuum-filtered as described above, and washed three times with ice-cold Tris-buffer. Filters were placed in liquid scintillation vials (LSV), 5 ml of Ready Gel™ (Beckman Coulter) was added, and the vials were capped and shaken. The activity of the ^{3}H-isradipine was determined by adding 10 μl of the radioligand to a LSV containing 5 ml of Ready Gel™ and reading it at the same time as the samples. The samples were left at room temperature overnight in minimal light, then counted in the β-counter (Beckman LS5000TA). The B_{MAX} and K_D were determined from the non-linear regression generated using GraphPad Prism3 software.

In order to determine the specific binding to the L-type channels in the SON and NH, a concentration of ^{3}H-isradapine 20 times the K_D determined from the saturation binding curve in

the whole brain (i.e. 1.6 nM) was used. Samples were prepared in a total volume of 80 µl with ~50 µg protein/assay. Specific binding was estimated as total binding minus nonspecific binding of the mean of triplicate assays.

RT-PCR

RT-PCR provides a means to examine the presence of specific gene products within a tissue sample or a single cell, and to quantify changes in transcriptional activity. In these studies, tissue homogenates of the SON from control and dehydrated rats were used. The SON was quickly isolated in either Tris-HCl or PIPES buffer and no consideration was given to the osmolality of the isolation buffer.

RNA Extraction. Tissue was placed in 1 ml of TriZol (Invitrogen) buffer (in a 1.5 ml Eppendorf tube), broken up by pipette mixing, incubated 5 min at 20ºC and 200 µl chloroform was added. Tubes were shaken for 15 seconds, allowed to sit for 2-3 minutes at 20ºC, and then centrifuged at 11,900 ×g, 4ºC for 15 min. The aqueous phase containing the RNA was decanted and 500 µl isopropyl alcohol was added and mixed by inverting. Tubes were incubated for 10 minutes at 20ºC, then centrifuged at 11,900 ×g, 4ºC for 10 min. To wash, the isopropyl alcohol was carefully poured off. One ml 75% ethanol was added and vortexed thoroughly, and centrifuged at 7,500 ×g, 4ºC for 5 min. The supernatant was poured off, the sample was allowed to air dry briefly, then 20 µl of RNAase free water was added and rinsed down thoroughly by pipette to dissolve the RNA. The sample was collected by a quick spin and stored at -70ºC.

First Strand cDNA Synthesis. This was done according to the kit (SuperScript™ First-Strand Synthesis System, Invitrogen) protocol.

PCR. Primer oligonucleotides for the α_1-subunits and GAPDH were synthesized at University Core DNA Services, University of Calgary, according to the primer sequences in Table 2.1

(Glasgow *et al.*, 1999). Table 2.2 gives the details of the PCR conditions used. All conditions were performed in triplicate.

Table 2.1. Primer sequences used

mRNA species	Sequence	5'-Position	PCR product (bp)
α_{1A} F	5'-CGAGCGGCTGGATGACACAGAACC-3'	396	421
α_{1A} R	5'-GAGCTGGCGACTCACCCTGGATGTC-3'	817	
α_{1B} F	5'-GAAGTAGCTGAAGTCAGCC-3'	2221	501
α_{1B} R	5'-CTTGTGTGTCAGCCCCTGGA-3'	2722	
α_{1C} F	5'-TCGTGGGTTTCGTCATTGTCA-3'	4236	474
α_{1C} R	5'-CCTCTGCACTCATAGAGGGAGAGG-3'	4710	
α_{1D} F	5'-GAGCCTGCATTAGTATAGTGAATTG-3'	873	217
α_{1D} R	5'-AGGATGCAGCAGCAGTCCGTA-3'	1090	
GAPDH F	5'-GGACATTGTTGCCATCAACGAC-3'	149	441
GAPDH R	5'-ATGAGCCCTTCCACGATGCCAAAG-3'	589	

Table 2.2. PCR cycle conditions

	Temp (ºC)	Time (min:sec)	
	95	3:00	
Denature	95	0:40	} Remove tubes at 3 cycle intervals starting at 23
Anneal	58	1:00	
Extend	72	1:30	
	72	5:00	

Tubes removed at the following points:

Primer	Cycle completed
GAPDH	23, 26, 29
α_1	30, 33, 36

Immunocytochemistry

Techniques for immunocytochemistry followed the methodology described by others (Oliet & Bourque, 1992; Fisher *et al.*, 2000). Briefly, tissue blocks containing the SON were harvested as above but in PIPES buffer (PIPES-Na 20 mM, NaCl 120 mM, KCl 5 mM, $MgCl_2$ 1 mM, pH

7.1) with glucose (25 mM) and $CaCl_2$ (1 mM) (PGC). Tissue blocks were placed in a glass tube containing PGC plus trypsin (Type XI, 0.6 mg/ml) and bubbled with 100% O_2 in a water bath at 34°C for 90 minutes. The tissue blocks were washed in trypsin-free PGC at room temperature and bubbled with 100% O_2 for 30 minutes. Tissues were triturated through a series of fire-polished Pasteur pipettes of decreasing diameter, plated on untreated glass-bottom culture dishes, covered and left undisturbed at room temperature for 1 hour. Debris was rinsed off with filtered PGC. The acutely isolated somata were rinsed twice with PBS and fixed for 20 minutes in PLP fixative (PBS with 4% paraformaldehyde, 0.05% sodium periodate, 0.34% lysine). Following eight rinses in PBS, the dishes were incubated 30 minutes in blocking solution (PBS with 4% normal goat serum, and 0.02% Triton X-100), and left overnight at 4°C in blocking solution containing the indicated antibodies. These included anti-α_{1B}, -α_{1C} (Alomone; 1:500) and -α_{1D} (Calbiochem; 1:500). Following eight rinses in blocking solution, the dishes were incubated for 30 min in the dark with an anti-rabbit secondary antibody from sheep (conjugated with Cy2 or Cy3, diluted 1:100; Amersham) The dishes were then rinsed four times in blocking solution, four times in PBS, filled with mounting solution (Citi-Fluor; Marivac), topped with a coverslip, and maintained at 4°C until viewing. Samples were viewed with the Zeiss Axiovert inverted microscope using a mercury lamp and images photographed using a CCD camera.

Data analysis

Quantitative data were entered in a GraphPad Prism or Excel worksheet and the Student t-test was used to determine statistical significance.

CHAPTER 3
RESULTS

Radioligand binding studies

To test whether dehydration induces a change in Ca^{2+} channel density in the osmoresponsive MNCs, radioligand binding studies were used. This type of study has proven to be an excellent tool for quantifying channel or receptor density within a tissue sample. Several pharmacological molecules specific to the N-, P/Q-, and L -type calcium channels can be iodinated or tritiated without altering their binding properties (Kristipati *et al.*, 1994). The ω-Ctx-GVIA ligand has high specificity and affinity for the N-type Ca^{2+} channels (Olivera *et al.*, 1991), and when iodinated at Tyr22, has been successfully used to measure channel density in whole chick brain (Ichida *et al.*, 1996), rat brain synaptic membranes (Jones & So, 1993) and rat brains (Qu *et al.*, 1998). Similarly, the $\alpha_{1C,D}$-subunits possess receptors for dihydropyridines, phenylalkamines and benzothiazepines. A nine amino acid residue in segments IIIS5, IIIS6 and IVS6 confers high affinity and stereoselective for DHPs (Berjukow *et al.*, 2000). ^{3}H-isradipine, one of the DHPs selective to the L-type Ca^{2+} channel, has been used to quantify channel density in rat heart, lung and brain (Nakayama *et al.*, 1999) and in rat brain and cultured neuronal cell homogenates (Hirota & Lambert, 1997).

^{125}I-ω-Ctx GVA binding to N-type Ca^{2+} channels

The dominant type of Ca^{2+} channel in the MNC somata and terminals is the N-type channel (Fisher & Bourque, 1996) and testing of the hypothesis began here. In order to quantify and compare the N-type channel density in the SON and NH, ^{125}I-ω-conotoxin-GVIA, a ligand known to specifically bind this channel was used. The mono-iodinated radioligand is labeled

with ^{125}I at Tyr^{22} and this does not interfere with the stereotaxic binding properties of the ligand (Cruz & Olivera, 1986). Concentrations of 0.005 – 0.6 nM of ^{125}I-ω-Ctx-GVIA, were used to generate saturating binding curves in pooled tissue homogenates of SON and NH. The B_{MAX} from the specific binding curve was obtained and the mean B_{MAX} for all experiments in each condition were compared using the Student t-test. As illustrated in Figure 3.1, when the individual experiments are analyzed and the mean B_{MAX} calculated, N-type Ca^{2+} channel density in the SON of control rats was 477±89 fmol/mg protein. Following the 16-24 hr period of water deprivation, channel density was 483±54 fmol/mg protein. This difference is not significant (p=0.5). Channel density was 413±61 fmol/mg protein in control NH and 379±56 fmol/mg protein in NH following the same period of water deprivation, and this decrease is not significant (p=0.3). All results are expressed as mean±se. The K_D was 0.079±0.008 nM (data not shown) which is within the range of that found by others for rat brain synaptic membranes of 0.1-0.3 nM (Jones & So, 1993) and 0.02±0.01nM (Qu *et al.*, 1998). As further confirmation that there was no change in N-type channel density, a comparison of the specific binding non-linear regressions, rather than the B_{MAX} value, was done using GraphPad Prism4. In Figure 3.2, the black line represents control values and the red line, values following the period of water deprivation. The dashed lines represent the 95% confidence interval for each condition and confirm that the B_{MAX} values for the N-type Ca^{2+} channels are not significantly different in the SON or NH of dehydrated animals.

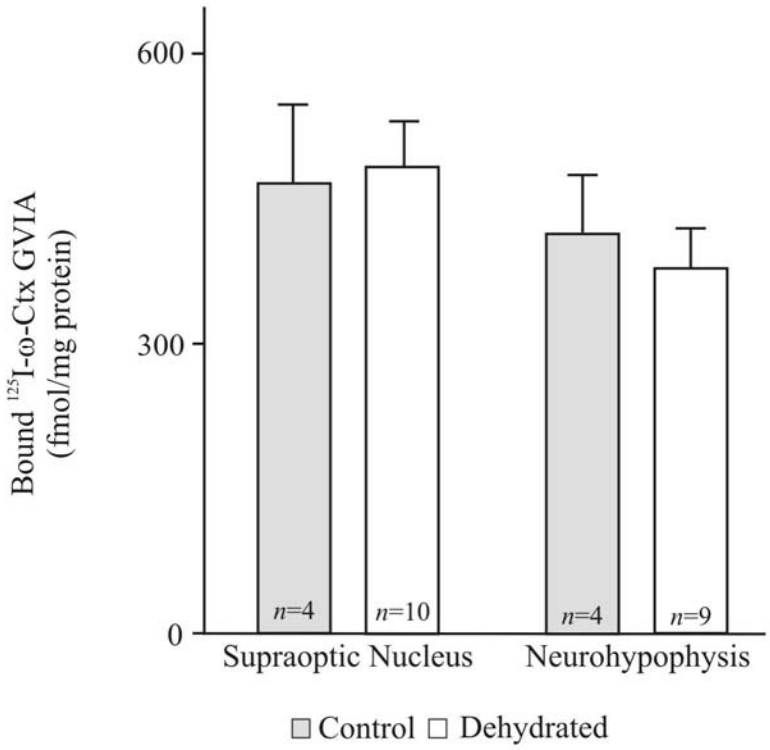

Figure 3.1. N-type Ca^{2+} channel density remains unchanged following a period of water deprivation. N-type Ca^{2+} channel density in the SON in control rats was 477±89 fmol/mg protein. Following 16-24 hours of water deprivation, this value was 483±54 fmol/mg protein, and this is not significantly different (p=0.5). Channel density was 413±61 fmol/mg protein in control NH and 379±56 fmol/mg protein in NH following the same period of water deprivation. This decrease is not significant (p=0.3). Data is shown as mean±se.

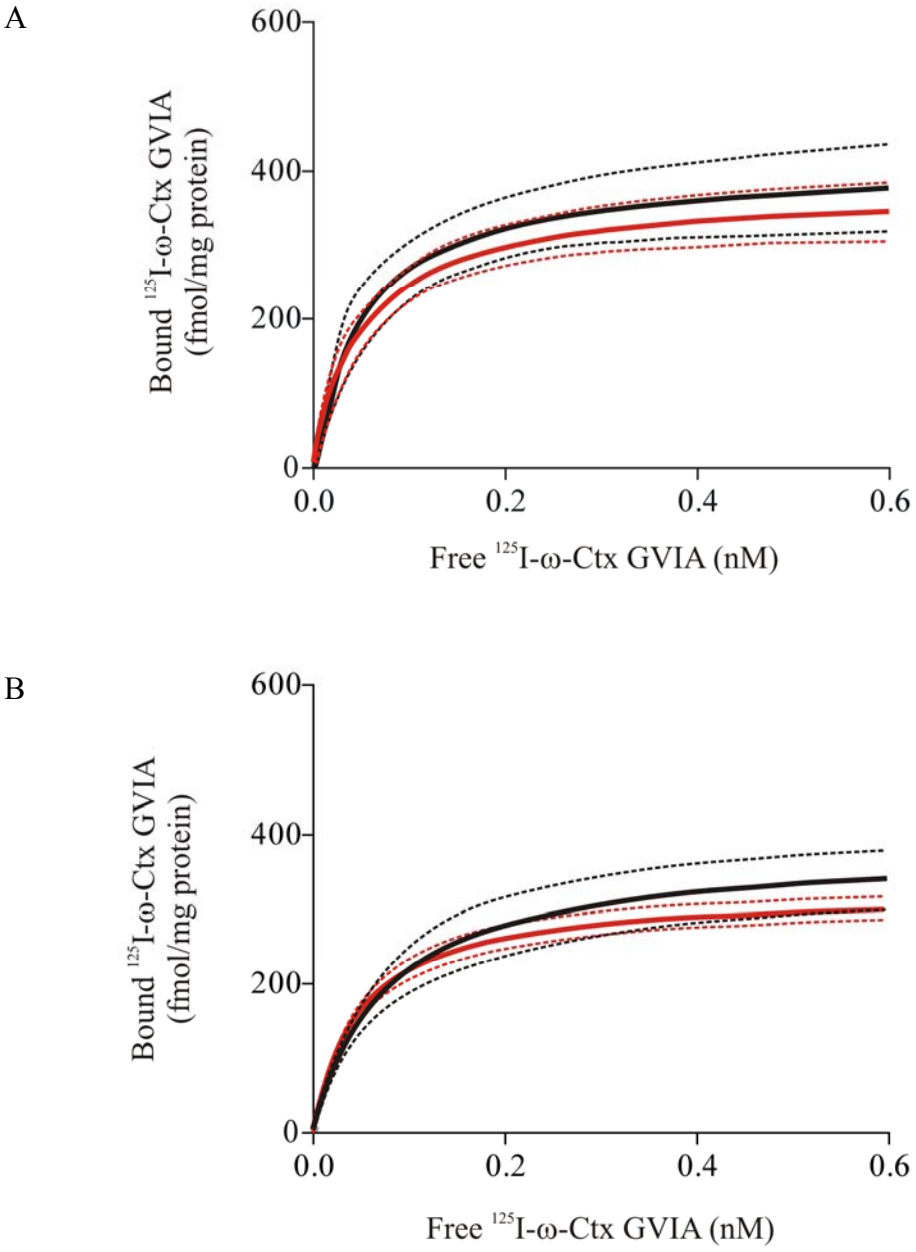

Figure 3.2. Specific binding to the N-type Ca^{2+} channels with ^{125}I-ω-Ctx GVIA. GraphPad Prism4 was used to analyze the specific binding for the ^{125}I-ω-Ctx GVIA to the N-type Ca^{2+} channels. The mean and standard error was calculated from a summary of all non-linear regression calculations, rather than the mean of individual experimental values for B_{MAX}. The resultant values were again analyzed by non-linear regression to give the graphs shown. Control values are shown in black and dehydrated in red. The dashed lines represent the 95% confidence level for each of the curves. The K_D is unchanged between all conditions. (A) The mean B_{MAX} for the dehydrated condition is not significantly different from the control condition in SON, although less than that calculated previous. (B) There is no difference in the mean B_{MAX} in NH, although also smaller than previously calculated.

^{125}I-ω-Ctx-MVIIC binding to P/Q-type Ca^{2+} channels

Changes in the P/Q-type Ca^{2+} channel density in the SON and NH could not be quantified due to the technical difficulties encountered with the only radioligand available for binding to this channel. The initial testing with ^{125}I-ω-Ctx-MVIIC was done in cortex and cerebellum, the latter chosen because of the known high density of the P-type channel. However, it was found that the non-specific binding in the presence of non-iodinated ω-Ctx-MVIIC represented as much as 80% of total binding, and it is recommended that non-specific binding be less than 50% at the highest concentration (Motulsky, 1999). Additionally, the ligand is known to have two binding sites, a high affinity site for the P/Q-type, and a low affinity site for the N-type channel (Kristipati *et al.*, 1994). Attempts were made to reduce the non-specific binding by pre-incubating the tissue with saturating concentrations of the N-type channel blocker, ω-Ctx-GVIA. However, this intervention failed to correct the problem, and obtaining reproducible data was elusive. Therefore, this research avenue was abandoned before testing of the SON and NH, where protein levels are much lower.

^{3}H-isradipine binding to L-type Ca^{2+} channels

The specific activity of ^{3}H-isradipine is low (86 Ci/mmol) and the amount of tissue in the SON and NH is relatively small (~55 μg SON and ~100 μg NH per animal). Therefore it was necessary to establish a saturating concentration of the ligand in rat brain tissue homogenates and use this value for a single-point assay on the SON and NH. Figure 3.3 is a representative graph of binding with ^{3}H-isradipine (0.05 to 1.6 nM) in rat brain. In the upper graph, the total binding is shown as black squares and the nonspecific binding as blue triangles. The latter represents binding of the tritiated ligand in the presence of a saturating concentration of nifedipine (1.5 μM)

and this binding is linear with respect to the concentration of ^{3}H-isradipine. The specific binding is calculated as the difference between total binding and the nonspecific binding, as shown in red in the lower graph. The non-linear regression of the resulting points provides a value for maximal density of binding sites for the ligand (B_{MAX}), and for the equilibrium dissociation constant (K_D) of the radioligand, where fractional occupancy is 50%. In rat brain, the B_{MAX} is estimated at 84.0±0.2 fmol/mg protein and the K_D at 0.089±0.001 nM. This value for K_D agrees with the value in cortex found by others of 88±12 pM (Hirota & Lambert, 1997). Based on these observations, a concentration of 1.6 nM ^{3}H-isradipine was used for the assays on the SON and NH, as it is approximately 20×K_D and therefore the fractional occupancy would be estimated at 95%. (Fractional occupancy = [Ligand]/([Ligand] + K_D) (Motulsky, 1999).

L-type Ca^{2+} channel density in the SON in control rats was 51.5±4.8 fmol/mg protein, n=8. Following 16-24 hours of water deprivation, this value was 68.1±4.1 fmol/mg protein, n=5. This 32% increase in channel density is significant (p<0.05). Channel density was 15.6±1.7 fmol/mg protein in control NH, n=10, and 21.6±5.0 fmol/mg protein, n=6, in NH following the same period of water deprivation. This increase is not significant (p=0.19) (Figure 3.4). Data is shown as mean±se.

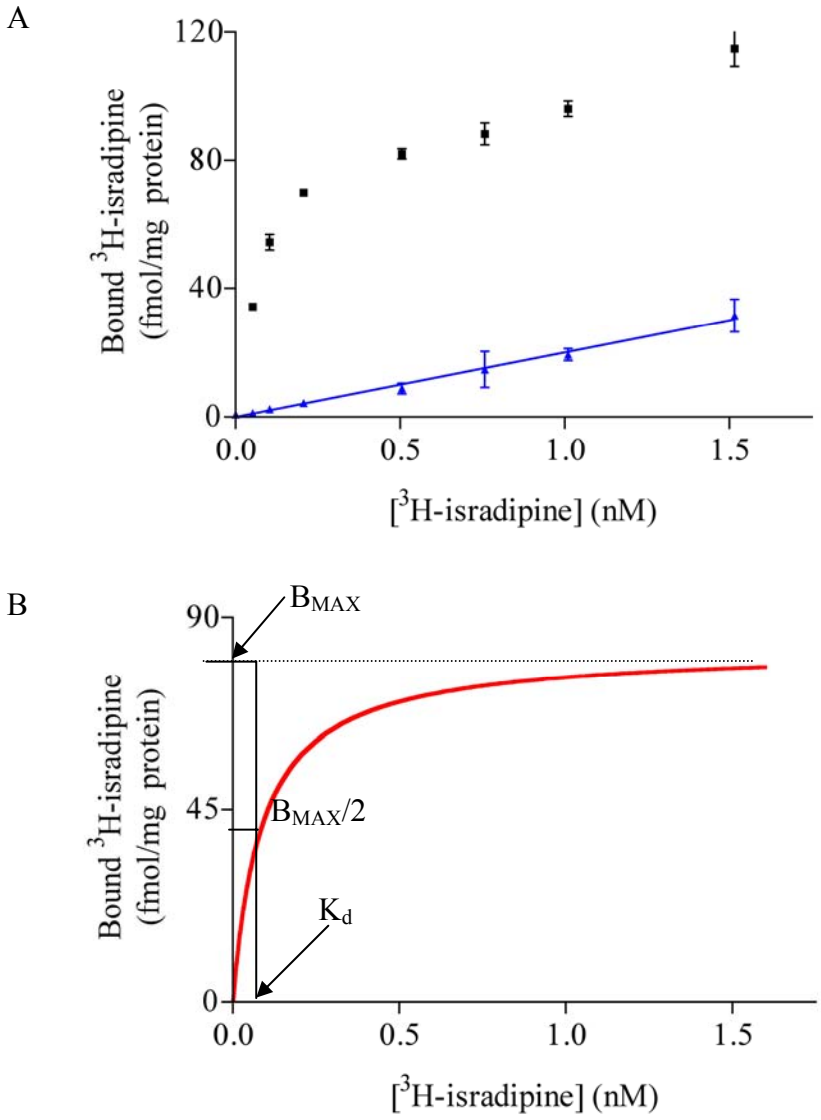

Figure 3.3. Determination of B_{MAX} for L-type channels in rat brain. (A) Saturation binding curves were obtained for ^{3}H-isradipine (0.05-1.6 nM), with total binding shown in the black squares and the nonspecific binding shown in the blue triangles. The latter represents binding of the tritiated ligand in the presence of a saturating concentration of nifedipine (1.5μM) and this binding is linear with respect to the concentration of the ^{3}H-isradipine. (B) The specific binding is calculated as the difference between total binding and the nonspecific binding, as shown in red. The non-linear regression of the resulting data points provides a value for maximal density of binding sites for the ligand (B_{MAX}), and for the equilibrium dissociation constant (K_D) of the radioligand, where fractional occupancy is 50%. From these studies, the B_{MAX} is estimated at 84.0±0.2 fmol/mg protein and the K_D at 0.089±0.001 nM. Based on these observations, a concentration of 1.6 nM ^{3}H-isradipine (approximately 20×K_D) was used for the single-concentration assay of SON and NH tissue samples.

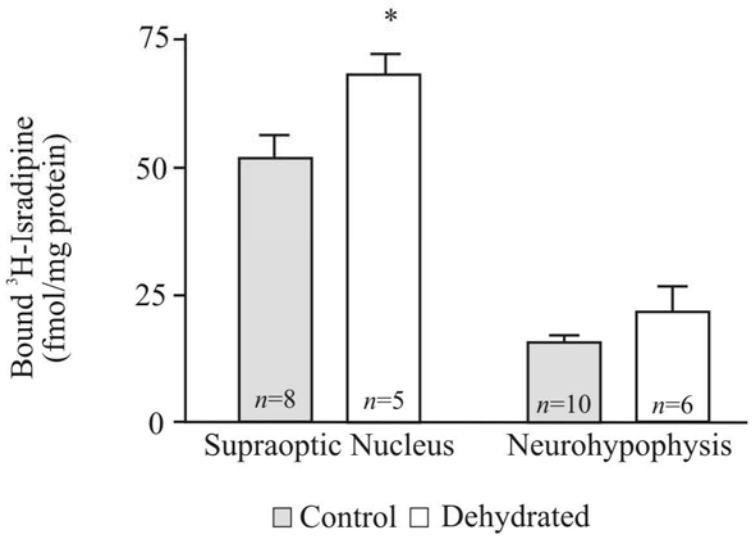

Figure 3.4. L-type Ca^{2+} channel density is increased in the SON following 16-24 hours of water deprivation. L-type Ca^{2+} channel density in the SON in control rats was 51.5±4.8 fmol/mg protein. Following 16-24 hours of water deprivation, this value was 68.1±4.1 fmol/mg protein. This 32% increase in channel density is significant (p<0.05). Channel density was 15.6±1.7 fmol/mg protein in control NH and 21.6±5.0 fmol/mg protein in NH following the same period of water deprivation. This increase is not significant (p=0.19). Data is shown as mean±se.

A single point comparison has its limitations and does not allow for the possibility that the affinity of the receptor for the ligand may have been altered by the treatment, ie. water deprivation. For example, the associated β-subunit is known to have an effect on the affinity of the receptor for the ligand (Pichler *et al.*, 1997), and dehydration could modify Ca^{2+} channel β-subunit activity. However, a change in affinity would have no effect on the B_{MAX} if the channels are saturated.

RT-PCR

RT-PCR provides a means to quantify changes in transcriptional activity. The mRNA was extracted from SON and NH tissue homogenates from single control and dehydrated rats, followed by first-strand cDNA synthesis. PCR using primers for GAPDH, a housekeeping gene,

was done in order to ensure that mRNA had been successfully extracted. Figure 3.5A shows that the PCR product is in fact expressed in all four tissue samples, and not in the negative control, which lacked cDNA. Further PCR reactions were restricted to the SON, site of the MNC nuclei, and cDNA from control and dehydrated rats was probed with primers for the α_{1B}-, α_{1C}-, and α_{1D}-subunits in parallel with GAPDH. Resultant PCR products were run on 2% agarose gels containing ethidium bromide, photographed under UV light and the photograph scanned. NIH ImageJ software was then used to compare the intensity of the bands. Each sample was prepared in triplicate so that PCR reaction could be stopped at 3-cycle intervals. This ensured that the reaction was in the exponential phase in all cases, and the mid-point was used for comparisons (see Figure 3.5B). Quantitative analysis was not done on α_{1C} because only one assay was carried out. Two distinct bands are observed for α_{1B}: the smaller, lower band represents the expected PCR product (Glasgow *et al.*, 1999), while the larger, upper one is likely an alternatively spliced variant resulting from the insertion of 21 amino acids in the II-III cytoplasmic loop of the channel protein (Pan & Lipscombe, 2000), an area bound by the primers used. Therefore, the possibility that expression of one or other variant could be influenced by the hydration status of the animal was considered. As shown in Figure 3.5C, no significant difference was found with the ratio to GAPDH being 0.66±0.05 vs 0.54±0.01 for the alternatively spliced variant, control vs dehydrated; and 0.46±0.03 vs 0.52 for the expected product. There was also no significant difference in expression of the α_{1D} product at 0.88±0.15 vs 0.73±0.09, control vs dehydrated, with n=3 for all conditions. This suggests that *de novo* synthesis is not required for the increase in L-type channel density.

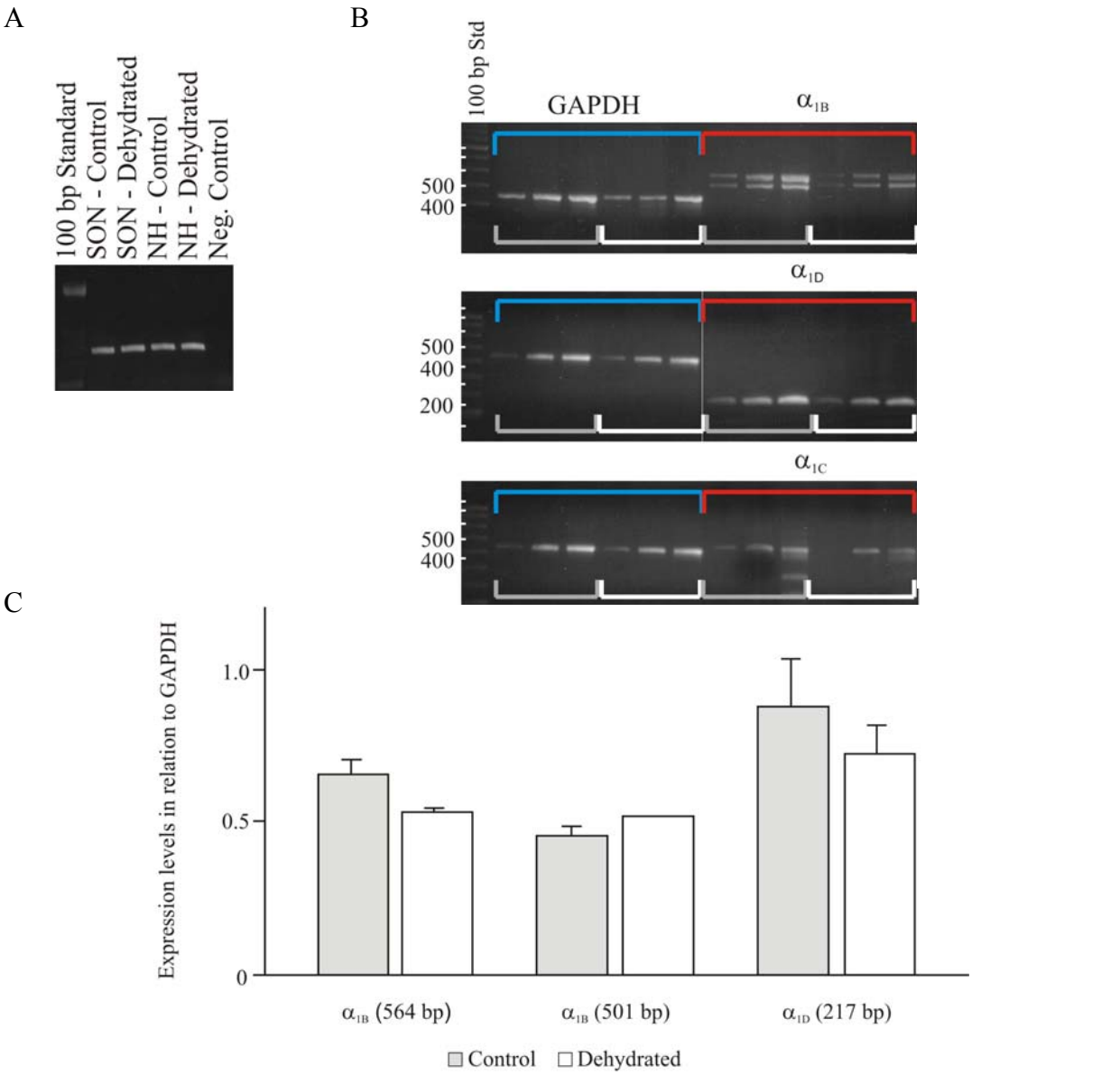

Figure 3.5. RT-PCR. Primers for GAPDH, α_{1B}, α_{1D} and α_{1C} were used on tissue homogenates of SON and NH from control and water-deprived rats. (A) GAPDH is present under all conditions, and not found in the negative control, which lacks template DNA. (B) PCR reactions were stopped at 3 cycle intervals and the mid-point compared to the intensity of the GAPDH band (under blue bar). The first three bands of each set (grey), represent the control and second (white), the dehydrated condition. It is thought that the second α_{1B} product is an alternatively spliced variant that includes an additional 21 bps within the II-III loop. (C) The ratio of the α_1-product intensity to GAPDH intensity shows no significant difference in control vs dehydrated, suggesting that *de novo* synthesis of the channels is not required for the observed increase in L-type channel density.

Immunocytochemistry

In order to determine if Ca^{2+} channels were differentially distributed between plasma membrane and cytosolic stores in the somata under control and dehydrated conditions, immunocytochemical analysis of isolated somata was carried out. Isolated somata were fixed and probed with antibodies against the α_{1B}-, and α_{1D}-subunits. Previous studies have demonstrated the presence of α_1-subunits internalized in the terminals, possibly associated with the dense core granules (Fisher *et al.*, 2000). No clear pattern of distribution of the specific α_1-subunit could be detected, and no comparison could be made between control and dehydrated somata. Therefore, this investigative approach to address the question of subunit distribution was not pursued.

CHAPTER 4
DISCUSSION

A water deprivation protocol was used to examine the stimulus-induced changes in Ca^{2+} channel density within the MNCs. Water deprivation increases the plasma osmolality in a time dependent manner and these changes can be quantified by measurements of the plasma osmotic pressure. In rat, the control value is 294 mOsmol/kg and increases to 306 at 6 hours, a 4.1% increase, and at 24 hours, to 340, a 15.6% increase (Wakerley *et al.*, 1978). Tightly correlated to this is a rise in the plasma concentration of VP due to the release of the hormone from the neurohypophysial terminals of the MNCs into systemic circulation. This increased hormone release is facilitated by changes in the firing pattern of the neurons. Accompanying the change in the firing pattern are significant ultrastructural changes within the SON and NH. The goal of this research project was to determine if a change in Ca^{2+} channel density could play a role in these modifications.

Changes in Channel Density

When measuring channel density using ligand binding studies, it is important that there is consistency of the K_D, as the affinity of the channel for the ligand can be modified by an associated subunit (Bichet *et al.*, 2000). It is possible that the β-subunit associated with a given α_1-subunit is changed by the treatment. Within the mammalian brain, the various β-subunits are present in different proportions suggesting that this association is important in the variability of channel properties (Mitterdorfer *et al.*, 1994; Pichler *et al.*, 1997). Other factors that can influence the K_D include the anesthetic halothane which has been shown to decrease the binding of isradipine in transverse tubule membrane of skeletal muscle by as much as 44% (Oz *et al.*,

2002) but since all rats received the same exposure to the halothane gas, this confounding factor was not considered. A change in the affinity would only generate an inaccurate B_{MAX} if equilibrium is not reached within the incubation period, or if non-saturating concentrations of the ligand are used.

Other researchers have shown that treatment of cells can lead to changes in channel density, measured either with binding studies or electrophysiologically. Following treatment that induces pulmonary hypertension, the density of the L-type channel is changed in tissue from lung and heart, while the K_D remained unchanged. The B_{MAX} decreased in the right ventricle and increased in the lung in the hypertensive rats (Nakayama *et al.*, 1999). Age-dependent changes in L-type channel density have also been observed in fetal and adult ovine cerebral and common carotid arteries with a decrease in B_{MAX} with maturation (Blood *et al.*, 2002). An increase in the N-type channels has been observed following kindling, an animal seizure model of epilepsy, suggesting that kindling alters N-type calcium channel trafficking mechanisms to cause a persistent, local remodeling of their distribution in hippocampal CA1 dendrites (Bernstein *et al.*, 1999). The insertion of N-type Ca^{2+} channels contributes to the synaptic potentiation at the synapse seen during kindling.

Studies that recorded Ca^{2+} currents in the SON are not as quantitative. Foehring and Armstrong (1996) found the DHP-sensitive current made up 28±2% of total I_{Ca}, and the ω-Ctx-GVIA-sensitive current was 26±4%, while Fisher and Bourque (1995) found these currents to be 23±2% and 39±3% respectively. Several considerations must be taken into account when interpreting these data, such as the charge carrier used (Ba^{2+} versus Ca^{2+}), as the single channel conductance of the L- and N-type channels differs, and also where the current was measured, whether it was peak or at the end of the stimulating pulse.

Based on the present binding study, the L-type channel represents a much smaller fraction of the Ca^{2+} channels present in the SON, approximately one tenth of the N-type. It was also observed that the density of the L-type channels in the NH was approximately 1/3 of their density in the SON. No significant change in L- or N-type Ca^{2+} channel density was noted in the NH, while there was a significant increase of the L-type and not the N-type in the SON. This raises a question as to the role that the L-type channel plays in the MNCs' response to changes in osmolality. Several possibilities come to mind including the role of Ca^{2+} in the generation of firing patterns, and in protein synthesis, as well as the important role Ca^{2+} influx plays in systemic and somatodendritic exocytosis of VP.

Since the N-type channel is a prominent Ca^{2+} channel in the MNCs, the investigations began with ^{125}I-ω-Ctx-GVIA, an iodinated ligand specific to this channel. Full saturation binding curves were generated and compared, and no change in the affinity (K_D) of the ligand for the receptor was found (see Figure 3.2). No change in the density of the N-type Ca^{2+} channel in the SON or the NH was observed, and it was concluded that dehydration does not induce a change in the density of this channel.

The DHP-sensitive L-type channels were then examined using ^{3}H-isradipine. As discussed in the Materials and Methods, the limited amount of protein available made it impractical to do a full saturation binding curve on the SON and NH. Therefore, a saturating concentration of ligand, calculated from the full binding curve on brain tissue, was used to determine the specific binding and the channel density. A single point comparison has its limitations and does not allow for the possibility that the affinity of the receptor for the ligand may have been altered by the treatment, in this case, water deprivation. In the present studies, it is believed that saturation of the L-type channels occurred and that the incubation period was sufficient. An increase in the

affinity can not cause a selective increase in the B_{MAX}, assuming that the channels are saturated at the concentration used.

To further test the hypothesis, electrophysiological studies were carried out in the lab (Star *et al.*, 2005). MNC somata were isolated by the same method used for immunocytochemistry. Cells isolated from control rats were maintained and recorded in whole cell mode in isoosmolar media and in hyperosmolar media, the latter to mimic a short-term exposure to dehydration. Cells from rats deprived of water for 24 hours were maintained in hyperosmolar media at every step of the isolation process, up to and including plating and recording from the cell in hyperosmolar media. Figure 4.1 shows that although the total Ca^{2+} current amplitude is not different in control and dehydrated rats, the amplitude of the nifedipine-sensitive current is significantly increased by dehydration, but not by short exposure to the hyperosmolar media. This finding supports the increase in L-type channel density but does not explain how the total current is unchanged. However, even a large increase in a small component of total current would be difficult to detect, and this requires further investigation.

The increase observed in the L-type channel density in the SON is likely an underestimation of the actual change. Although the SON consists of the densely packed osmosensitive MNC somata (Sladek, 2004) the tissue sample collected also contains membrane from glial cells and afferent neurons. These cells contain Ca^{2+} channels that are not likely to be affected by changes in osmolality. The density of the latter would likely remain constant despite the treatment, and this background amount was not subtracted from the total when determining the percent change.

Given that a significant increase in the L-type Ca^{2+} channel density was seen, the next question to ask is where do these channels come from? Several possibilities come to mind

including the synthesis of new channels, the activation of pre-existing membrane channels and the translocation of channels from internal stores to the membrane.

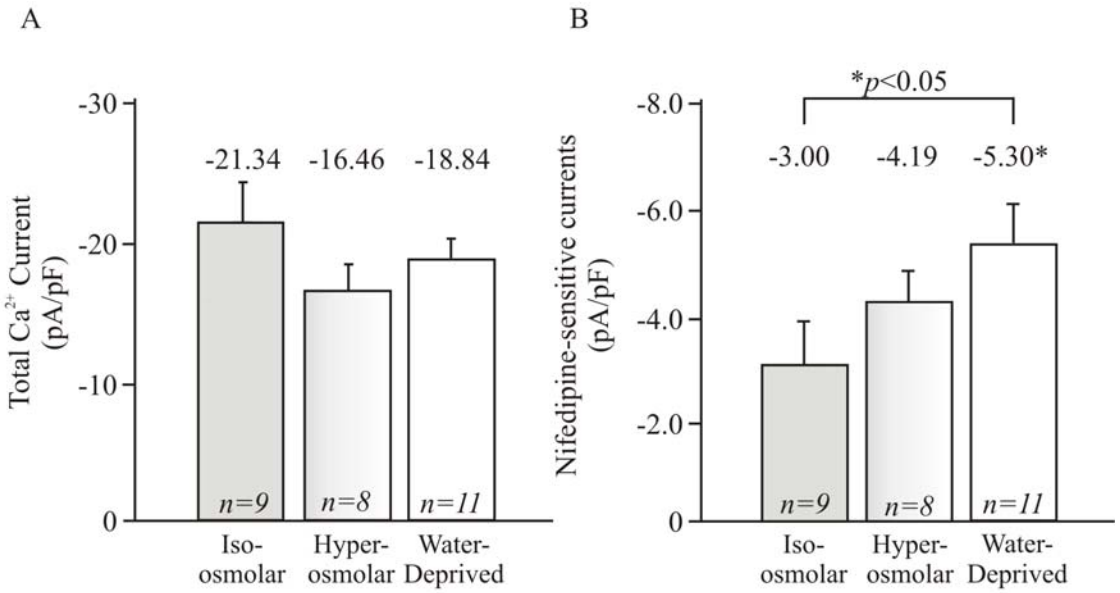

Figure 4.1. Ca^{2+} currents in isolated MNC somata. Total Ca^{2+} current amplitude is not different in MNC somata isolated from control and dehydrated rats, while the amplitude of the nifedipine-sensitive current is increased by dehydration. Isolated MNC somata were examined under three conditions. Iso-osmolar conditions had cells from control rats plated in media at 295 mOsmol/kg. Hyper-osmolar conditions were induced by taking normal cells, prepared as previously described and plating for 1-2 hours in media modified by the addition of mannitol to an osmolality of 325 mOsmol/kg. Cells isolated from dehydrated rats were maintained in hyper-osmolar media (325 mOsmol/kg) throughout. (A) Bar graphs show the mean ± standard error of total Ca^{2+} current amplitude evoked by a step from a holding potential of -100 mV to a test potential of -10 mV. (B) Nifedipine-sensitive currents are significantly increased in isolated cells from dehydrated rats. (Star *et al.*, 2005)

Synthesis of new channels

The possibility of new channel synthesis was examined using RT-PCR. If these observed channels were newly synthesized, one would expect to see an increase in the expression levels of the mRNA for the α_{1C}- and/or α_{1D}-subunits, the L-type channels expressed in the brain (Lipscombe *et al.*, 2004). The expression levels for α_{1C} were very low; therefore the focus was

on the α_{1D}, which is the dominant L-type channel in the MNC soma (Glasgow *et al.*, 1999). No significant difference in the expression levels of the PCR products was detected, which suggests that *de novo* synthesis of channels is not increasing, at least over the short term of 24 hours of water deprivation. Two PCR products were observed with the primers for α_{1B}, the second likely an alternatively spliced variant of the α_{1B}-subunit (Pan & Lipscombe, 2000). However, only sequencing of the products could confirm this.

Activation of pre-existing membrane channels

Another possible explanation for this observation is that the channel did not change in number or density but rather in its ability to bind the ligand. Dehydration could induce the post-translational modification of pre-existing channels through second messenger pathways that could include phosphorylation, dephosphorylation, or glycosylation. As discussed above, it is also possible that there is a change in the associated β-subunit, and this could affect the probability of the channel being activated. Although the channels possess a receptor within the pore region for the DHPs, isradipine and nifedipine are not endogenous molecules. They bind with high affinity to the inactivated state of the α_1-subunit of DHP-sensitive channels (Berjukow *et al.*, 2000). It is possible that the state of the channel will affect the probability of binding but unlikely to account for the increase observed in the L-type channel density seen only in the SON and not the NH.

Translocation of channels from internal stores to the membrane

A third possible explanation for the observed increase in the L-type Ca^{2+} channels is that these proteins were previously synthesized and maintained in intracellular stores until stimulus-dependent insertion occurred. This translocation provides an immediate source of channels and it is used in physiological systems. In sea urchin eggs, there are no P-type Ca^{2+} channels present on

the egg surface prior to fertilization, but they appear immediately after egg activation (Smith *et al.*, 2000). The channels reside on cortical granule membranes before fertilization, and depolarization-induced exocytosis of Ca^{2+} channel-containing cortical granules at fertilization creates a necessary physical barrier that prevents polyspermy. The exocytotic event significantly increases the cell surface area, an undesirable side effect, and this increase is quickly remedied by endocytotic retrieval of the membrane at sites of previous exocytosis. In other words, exocytosis transiently inserts Ca^{2+} channels into a depolarized plasma membrane, and subsequent Ca^{2+} influx through these channels causes selective recovery of the cortical granule membrane in which it resides.

Bag cell neurons of *Aplysia californica* also have an internal store of Ca^{2+} channels (White & Kaczmarek, 1997). These neurons secrete egg-laying hormone (ELH), the peptide responsible for the initiation of egg-laying behaviour during the afterdischarge, a prolonged period of electrical activity. Two distinct Ca^{2+} channels are found on these cells, differentiated based on distribution, sensitivity to protein kinase C (PKC), and unitary conductance. Both are high voltage activated, slowly inactivating, and sensitive to high DHP concentrations and therefore show considerable similarity to L-type Ca^{2+} channels. The BCCa-I subtype appears to be expressed exclusively in neurons while the BCCa-II is found in neurons, as well as glia and muscle. Within the neurons, the BCCa-I is associated with the vesicles, although not those containing the ELH, while the BCCa-II is uniformly distributed in the membranes of the bag cell neurons. There are also two Ca^{2+} currents, one that seems to underlie the Ca^{2+}-based action potentials evoked from bag cell neurons by electrical stimulation. Only after stimulation of PKC is the second current detectable and it appears to mediate the enhancement of action potentials and ELH release during the afterdischarge. The stimulation of PKC appears to recruit the internal

BCCa-I channels to the plasma membrane (Strong *et al.*, 1987). In addition, these channels may reside on Ca^{2+}-containing vesicles and therefore facilitate Ca^{2+}-release and sustenance of ELH release (White *et al.*, 1998).

As discussed in the Introduction, in pheochromocytoma cells (PC12) "hidden" voltage dependent Ca^{2+} channels are present in the membrane of intracellular secretory granules (Passafaro *et al.*, 1996). Exocytosis causes these channels to be reversibly inserted in the plasma membrane at the same time as the vesicular contents are released into the extracellular space. In RINm5F cells, the insulin- and serotonin-secreting endocrine cells that express the N-type Ca^{2+} channel, the internally stored channels participate in the potentiation of intracellular Ca^{2+} transients seen during repetitive depolarizations (Passafaro *et al.*, 2000). A strong correlation was demonstrated between hormone secretion and voltage-activated Ca^{2+} channel recruitment that was not simply a modification of preexisting channel gating properties. In these cells, stimulated secretion is always associated with channel recruitment, while inhibition of secretion prevents voltage-activated Ca^{2+} channel recruitment. The implication of this adaptation is that it enables plasticity of the secretory process as well as other calcium-dependent events such as second-messenger pathways of cellular activation.

Translocation of a growth-factor-regulated channel (GRC), to the plasma membrane occurs in cells exposed to insulin-like growth factor-I (IGF-I). This channel is a member of the transient receptor potential (TRP) family of signal-transduction-gated nonselective cation channels that are thought to be involved in the replenishment of intracellular Ca^{2+} stores. TRP channels are localized mainly to intracellular pools under basal conditions, and the IGF-I supplements Ca^{2+} entry by regulating trafficking of the channel (Kanzaki *et al.*, 1999). Another observation that supports translocation as a mechanism by which the Ca^{2+} current is modified is the role of Ca^{2+}-

activated K^+ channels, possibly a TRPC5 channel. This channel is transiently inserted into the membrane from quiescent internal storage locations following growth factor stimulation of HEK-293 cells, (Bezzerides *et al.*, 2004). The channels are thought to be sequestered in vesicles and inserted into the plasma membrane when and where they are needed. This process also occurs in hippocampal neurons at the sites of growth cone extension. The rapid insertion and retrieval of the TRP channels may be a cellular response to stimuli that affect cell morphology and/or direction of movement and provide a mechanism for transient, self-limiting increases in calcium influx.

Translocation has been demonstrated in the MNCs where the κ-opioid receptor (KOR1) is reversibly translocated to the synaptic plasma membrane of the VPergic terminals from peptide-containing vesicles following a stimulus for VP secretion (salt-loading) (Shuster *et al.*, 1999). Dynorphin is co-released with VP in the terminals (Whitnall *et al.*, 1983) and will be available in the extracellular space for binding the receptor. κ-opioid agonists decrease calcium conductance in the neural lobe resulting in inhibition of peptide release. Therefore, the increase in KOR1 receptor binding following stimulation would provide a means of modulating release and the regulated exocytosis serves to bring secreted peptides and proteins to the extracellular space together with plasma membrane receptors necessary to modulate neuropeptide release.

When the channels observed on internal granules are translocated to the plasma membrane following stimulation, this could have many implications on the functioning and control of the secretory process.

Physiological significance of the increased L-type channels

An increase of 32% in the channel density of a specific Ca^{2+} channel subtype suggests that the voltage-dependent L-type Ca^{2+} channel plays an important role in the response of the MNC to

dehydration, and this function is specific to the somata. If this increase was important to hormone release *per* se, one would expect to see a larger increase at the dominant site of release, the terminals, and this was not the case. There is no increase in the density in the terminals. In the somata, the increase could involve changing the membrane electrophysiological properties, with consequent modifications in the firing pattern to maximize systemic release. An increase in the number of MNCs that burst or optimization of their bursting behaviour could be mediated by the entry of Ca^{2+} through the newly activated channels. There are several possible mechanisms by which this could occur, including increasing the activation of Ca^{2+}-activated K^{+} channels, which play a role in bursting (Shevchenko *et al.*, 2004) or by increasing the size of any other Ca^{2+}-dependent current, such as the depolarizing after potential (DAP).

Calcium is critical for many of the second-messenger pathways, and the L-type channel is directly involved in the activities of gene transcription and translation in the somata (Burbach *et al.*, 2001). Therefore, an increase in Ca^{2+} influx could increase the synthesis of VP and OT, or any of the other proteins synthesized such as the V_{1a} receptor whose mRNA levels are increased in dehydration (Hurbin *et al.*, 2002). In this way, the reserve pool of VP and OT granules could be maintained as synthesis and trafficking of the hormone to the terminals could begin immediately upon the stimulation of the cell. The elevation in VP mRNA levels upon rehydration persists for a period of weeks, long after the need for increased release has been satisfied (Zingg *et al.*, 1986). It would be interesting to see if the increase in the L-type channel density also persists during this period.

If a function of increased channel density is the enhancement of somatodendritic release, then the actions of the hormones on their central receptors would also be enhanced. Excitatory and inhibitory neuronal inputs are influenced by the binding of VP and OT to their receptors and this

will impact further release of hormone (Di & Tasker, 2004). Vasopressin is known to enhance dendritic release but it cannot produce priming, which is the Ca^{2+}-evoked preparation of the releasable pool of VP-containing granules for release following depolarization-dependent activation (Ludwig *et al.*, 2005). The somatodendritic release of VP and OT is important, and the presence of additional Ca^{2+} channels could enhance this release and be part of the MNC's response to dehydration.

One question that remains unanswered is why an increase is observed in the density of the L- and not the N-type channel. One possible explanation is in the numbers themselves. The N-type represents a much larger proportion of the channels in the somata and therefore a small change may not be detectable. However, because there are many fewer L-type channels, a few more channels represent a significant increase, in this case, a 32% increase with the addition of 17 fmol/mg protein in the SON of the dehydrated rat. The L-type channels may be selectively localized or associated with specific proteins in order to carry out a specific function. Therefore, to affect a significant increase in that specific function, it is logical to have a small basal density of the channel and increase it markedly when the need arises. It is feasible that translocation of the L-type channel is a mechanism for the MNCs to moderate their activity in response to the physiological stress of dehydration.

Limitations of this study

The primary limitation of this study is that one cannot be sure that the changes in channel density were restricted to the MNCs and not inclusive of glia. Glial cells express Ca^{2+} channels in astrocytes, but not in oligodendrocytes or microglia (Barres, 1991), and it is the former that are present in the SON. A second uncertainty is whether the increase is due to translocation of channels as there is no evidence in the literature that translocation of these particular channels actually occurs in the MNCs.

The studies began with a water-deprivation period of 16 hours, which was subsequently increased to 24 hours. It is believed that this is enough time to test the hypothesis, without causing the rats excessive discomfort. It is not known if a longer period would result in further adaptive changes in the MNCs, or if the added stress experienced by the rats would initiate a tertiary response cascade.

Immunocytochemical analysis can be used to visualize the intracellular stores of Ca^{2+} channels, and an attempt was made to replicate this. However, it was not possible to visualize changes in channel distribution in any quantifiable way. This could be due to the small number of channels present, the lack of specificity of the antibodies for the α_1-subunits or the diffuse distribution of the channels throughout the cell.

Future directions

Although an increase in the number of L-type channels was observed and quantified, it is not certain that this increase is due to the presence of more channels on the plasma membrane of the MNC somata. Biotinylation is a technique that can differentiate between MNC somata plasma membrane proteins and those on internal stores or contributed by extraneous tissue. Isolated somata, prepared as for immunocytochemistry and electrophysiology, are incubated with a biotin-containing buffer. Biotin is chemically modified such that, in the presence of a catalyst, it will interact with the surface proteins, and when the cells are lysed and run through an avidin column, only the surface proteins will be collected due to the tight association between the biotin and avidin. All other proteins will be in the effluent. One can then take the biotinylated sample and the effluent and run them on gels. These Western blots could be probed with antibodies for the various α_1-subunits to see if there is a change in the distribution of the proteins between the MNC somata in control and dehydrated rats. Therefore, these experiments could provide a means

to substantiate our claim that the channels are translocated from internal stores to the membrane in a regulated fashion following a period of dehydration.

CHAPTER 5
CONCLUSION

Regardless of the triggering event, changes in plasma osmolality are rapidly compensated for by the secretion of VP. This hormone, when released into systemic circulation from the MNC terminals in the NH, acts on receptors in the kidneys to facilitate water reabsorption and the subsequent return to a normal state of hydration. It is also released from the somata and dendrites within the supraoptic nucleus where its role is less well understood. Exocytosis is always correlated to Ca^{2+} influx and therefore this study investigated if changes in Ca^{2+} channel density could play a role in the modification of the MNC properties following a period of chronic stimulation. It has been demonstrated here that the density of the L-type Ca^{2+} channel is increased significantly in the somata but not the terminals, while no change occurs in the N-type channel density. At the same time, there is no change in the total Ca^{2+} current but a significant increase in the nifedipine-sensitive current. Therefore, it appears that the L-type channel plays an important role in the MNC response to dehydration, and it is possible that this increase is the result of translocation to the somatodendritic plasma membrane of pre-existing cytosolic channels associated with the neuropeptide-containing granules. This research has demonstrated for the first time that the density of a specific Ca^{2+} channel is increased in the SON following a period of water deprivation.

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